CHARACTERISATION AND IDENTIFICATION OF THE ACTIVE MICROBIAL CONSORTIUM PRESENT IN KEPI GRAINS

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that it has not previously in its entirety or in part, been submitted at any university for a degree.

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ABSTRACT

Kepi is an acidic, self-carbonated milk beverage that is produced by fermenting milk with grain-like structures that contain naturally occurring microbes, including lactic acid bacteria (LAB) and yeasts. The specific microbes present in the Kepi grains are responsible for an acidic-alcoholic fermentation of the milk and also contributes to the various health properties exhibited by Kepi. The combination of microbes in the Kepi grains can vary considerably depending on which type of milk is fermented, the method by which Kepi is produced, the origin of the grains and how the grains are stored.

In this study, the impact of various environmental conditions including the different stages during Kepi production, grain origin, lyophilisation and packaging in three different packaging materials, on the microbial community of Kepi grains were studied using selective growth media, morphology and biochemical characteristics. It was found that there was a general decrease in the microbial counts from laboratory produced Kepi grains, the longer Kepi was produced on a continuous basis. This decrease in microbial counts was also observed during the different stages of Kepi production. The average LAB counts obtained from laboratory produced grains decreased from 1.1×10^8 cfu.g^{-1} after 3 d of activation to 6.3×10^7 cfu.g^{-1} after 10 d of mass production to 9.7×10^6 cfu.g^{-1} after a further 30 d of normal Kepi production. The average yeast counts increased from no detectable yeasts after 3 d of activation to 5.7×10^7 cfu.g^{-1} after 10 d of mass production and then decreased again to 7.2×10^6 cfu.g^{-1} after 30 d of normal Kepi production. The combination of the isolates varied according to the method by which the Kepi grains were produced and the stress conditions that were applied. Laboratory produced Kepi grains contained the following LAB: *Lactobacillus fermentum*, *Lb. brevis 3*, *Lb. plantarum*, *Lb. delbrueckii* subsp. *delbrueckii*, *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides* subsp. *cremoris*. The identified yeasts and mycelial fungi were a *Zygosaccharomyces* strain, *Cryptococcus humicolus*, *Candida lambica*, *C. krusei*, *C. kefyr* and *Geotrichum candidum*.

The influence of grain origin on the microbial content of Kepi grains was also investigated using samples of Kepi grains from eight different Southern African sources. The microbial counts of the various Kepi grain samples were

found to vary from 6.0 x 10^5 cfu.g^{-1} to 1.7 x 10^8 cfu.g^{-1}. Five *Lactobacillus*, two *Leuconostoc*, four *Candida*, one *Saccharomyces* and a *Zygosaccharomyces* strain were isolated from these grains, with each grain type having its own unique microbial combination.

The microbial content of the Kepi grains that were lyophilised, packaged in three different packaging materials and stored at room temperature for two months, was very similar. *Lactobacillus delbrueckii* subsp. *delbrueckii* was isolated from the Kepi grains packaged in "low density polyethylene film" (LDPE). The grains packaged in "oriented polyester film" (OPET) contained *Lb. delbrueckii* subsp. *delbrueckii* and *Lb. brevis*, while *Lb. delbrueckii* subsp. *delbrueckii* and *Lb. curvatus* was present in the grains packaged in "methallised oriented polyester film" (MOPET). The average microbial counts obtained from the Kepi grains packaged in OPET (2.7 x 10^6 cfu.g^{-1}) were only slightly higher than that of the grains packaged in LDPE (1.2 x 10^6 cfu.g^{-1}) and OPET (1.4 x 10^6 cfu.g^{-1}). It was concluded that packaging materials for Kepi grains should rather be evaluated on the quality of Kepi produced with the packaged grains than by the specific characteristics of the packaging materials.

The enrichment of Kepi grains with propionibacteria was also evaluated. A polymerase chain reaction (PCR) based method, specifically designed for the rapid identification of propionibacteria, was developed and tested successfully. Using this technique it was concluded that propionibacteria were not a natural part of the Kepi beverage and grains as used in this study. However, during the enrichment of the grains with propionibacteria it was determined that a propionibacteria concentration of 1 x 10^8 cfu.ml^{-1} was needed for successful PCR amplification results.

The data obtained in this study clearly showed that the method by which Kepi is produced, the origin of Kepi grains and the method of Kepi grain preservation changes the relationship between the microbes constituting the grains to such an extent that a different microbial community is assembled. It was also concluded that traditional methods should be used together with newer methods in determining this microbial community.

UITTREKSEL

Kepi is 'n self-gekarboneerde, effens suur melkdrankie wat geproduseer word deur melk te fermenteer met korrels waarin mikrobes (melksuurbakterieë en giste) natuurlik voorkom. Die mikrobes in die Kepi korrels is verantwoordelik vir 'n suur-alkoholiese fermentasie en dra verder by tot die verskeie gesondheidseienskappe wat Kepi besit. Die kombinasie van mikrobes in die Kepi korrels wissel afhangende van die tipe melk wat gebruik word, die metode waarvolgens Kepi gemaak word, die oorsprong van die korrels en hoe die korrels geberg word.

In hierdie studie is die impak van verskeie omgewingskondisies insluitende die verskillende stadiums tydens Kepi produksie, korrel oorsprong, vriesdroging en verpakking in drie verskillende verpakkingsmateriale, op die mikrobiese samestelling van Kepi korrels bepaal m.b.v. selektiewe groei media en morfologiese en biochemiese eienskappe. Dit is gevind dat daar 'n afname was in die mikrobiese tellings van laboratorium geproduseerde Kepi korrels hoe langer Kepi op 'n aaneenlopende basis geproduseer is. Die afname in mikrobiese tellings is ook waargeneem tydens die verskillende stadiums van Kepi produksie. Die gemiddelde melksuurbakterieë tellings van laboratorium geproduseerde korrels het afgeneem vanaf 1.1 x 10^8 kve.g^{-1} na 3 d van aktivering tot 6.3 x 10^7 kve.g^{-1} na 10 d van massakweking tot 9.7 x 10^6 kve.g^{-1} na 'n verdere 30 d van normale Kepi produksie. Die gemiddelde gis tellings het gestyg vanaf geen giste na 3 d van aktivering tot 5.7 x 10^7 kve.g^{-1} na 10 d van massakweking en het toe weer gedaal tot 7.2 x 10^6 kve.g^{-1} na 30 d van normale Kepi produksie. Die kombinasie van die isolate het gewissel na gelang van die metode waarop die Kepi korrels geproduseer is en die stres kondisies wat toegepas is. Laboratorium geproduseerde Kepi korrels het bestaan uit *Lactobacillus fermentum*, *Lb. brevis 3*, *Lb. plantarum*, *Lb. delbrueckii* subsp. *delbrueckii*, *Lactococcus lactis* subsp. *lactis 1* en *Leuconostoc mesenteroides* subsp. *cremoris*. Die giste en misiliëre fungi wat geïsoleer is was 'n *Zygosaccharomyces* stam, *Cryptococcus humicolus*, *Candida lambica*, *C. krusei*, *C. kefyr* en *Geotrichum candidum*.

Die invloed wat die oorsprong van Kepi korrels op die mikrobiese samestelling daarvan het, is bepaal m.b.v. Kepi korrels afkomstig van agt verskillende dele in Suidelike Afrika. Die mikrobiese tellings van die verskeie tipes Kepi korrels het gewissel vanaf 6.0 x 10^5 kve.g^{-1} tot 1.7 x 10^8 kve.g^{-1}. Vyf

Lactobacillus, twee *Leuconostoc*, vier *Candida*, een *Saccharomyces* en 'n *Zygosaccharomyces* is geïsoleer vanuit die korrels, waarvan elke tipe korrel sy eie unieke mikrobiese samestelling gehad het.

Die mikrobiese samestelling van korrels wat gevriesdroog, verpak is in drie verskillende verpakkingsmateriale en by kamertemperatuur gestoor is vir twee maande, was baie eenders. Vanuit die Kepi korrels wat verpak is in "lae digtheid polietileen film" (LDPE) is *Lb. delbrueckii* subsp. *lactis* geïsoleer. Die korrels wat verpak is in "georienteerde polïester film" (OPET) het *Lb. delbrueckii* subsp. *lactis* en *Lb. brevis* besit, terwyl *Lb. delbrueckii* subsp. *lactis* en *Lb. curvatus* teenwoordig was in die korrels wat in "gemetileerde georienteerde polïester film" (MOPET) verpak is. Die gemiddelde mikrobiese tellings van die korrels wat verpak is in OPET (2.6×10^6 kve.g^{-1}) was effens hoër as dié van die korrels wat verpak is in LDPE (1.2×10^6 kve.g^{-1}) en MOPET (1.3×10^6 kve.g^{-1}). Dit is bepaal dat verpakkingsmateriale vir Kepi korrels eerder geevalueer moet word op die kwaliteit van die Kepi wat met die verpakte korrels geproduseer word, as op die spesifieke eienskappe van die verpakkingsmateriale.

Die mikrobiese verryking van Kepi korrels met propionibakterieë is ook ondersoek. 'n Polimerase ketting reaksie (PKR) gebaseerde metode, spesifiek ontwerp vir die vinnige identifikasie van propionibakterieë, is ontwikkel en suksesvol getoets. Met hierdie tegniek is bepaal dat propionibakterieë nie 'n natuurlike deel is van die Kepi drankie en korrels soos gebruik in hierdie studie. Gedurende die verryking van Kepi korrels met propionibakterieë is dit egter ook bepaal dat 'n propionibakterieë konsentrasie van 1×10^8 kve.ml^{-1} nodig is vir suksesvolle PKR amplifikasie resultate.

Die data verkry in hierdie studie het duidelik gewys dat die metode van Kepi produksie, die oorsprong van Kepi korrels en die metode waarop Kepi korrels gepreserveer word, verander die verhouding tussen die mikrobes in die korrels tot so 'n mate dat 'n nuwe mikrobiese gemeenskap saamgestel word. Die gevolgtrekking is ook gemaak dat tradisionele metodes saam met nuwer metodes gebruik moet word in die bepaling van hierdie mikrobiese gemeenskap.

To my father and late mother, for their endless love and support

"An university education should equip one to entertain three things: a friend, an idea and oneself".

-Thomas Ehrlich-

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CONTENTS

Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

Fermented products have been developed throughout the world as a means of preserving milk against spoilage, as well as to provide a greater variety of foods (Litopoulou-Tzanetaki & Tzanetakis, 1999). In addition to the purely nutritional value of fermented milk products, they also possess many therapeutic and antimicrobal properties and are widely used to cure certain metabolic and stomach disorders (Gurr, 1987; Oberman & Libudzisz, 1998;). The fermented milks include many commercial products such as buttermilk, yoghurt and sour cream (Robinson, 1999; Vedamuthu, 1982), as well as many traditional products such as Leben (Iraq, Lebanon), Dahi (India), Kepi (Caucasus) and Koumiss (Kazakhstan, Mongolia) (Koroleva, 1988; Özer & Özer, 1999). Both the commercial and traditional fermented milk products involve the inoculation of milk with microbial cultures. Commercial products are inoculated with pure cultures, while traditional products, such as Kepi, are produced using natural occurring complex microbial populations (Koroleva, 1988).

Kepi is a fermented milk beverage that originated in Russia and has been consumed for thousands of years (Kwak *et al.*, 1996). The traditional production of this product involves the fermentation of milk with grains consisting of a consortium of bacteria and yeasts that exist in a complex symbiotic relationship. The Kepi grains are small, unevenly shaped and cream to white in colour (Roginski, 1988) and the complex microbial population that the grains are composed of, are held together by a polysaccharide matrix called kefiran (Steinkraus, 1996).

It is difficult to determine the exact number of microbes present in the Kepi grains. As many are not viable outside the Kepi grain environment and, in addition, it is difficult to separate the microbes from the kefiran matrix which further complicates the isolation of the microbial community (Garrote *et al.*, 1997). The average concentrations of the different microbes in the Kepi grains have been reported to be between 10^3 – 10^9 cfu.g^{-1} bacteria and 10^4 – 10^8 cfu.g^{-1} yeasts (Garrote *et al.*, 1997; Litopoulou-Tzanetaki & Tzanetakis, 1999; Pintado *et al.*, 1996).

The Kepi grains also constitute many different microbial species that can vary greatly depending on the type of milk used, the origin and storage of the Kepi grains and the production method followed (Garrote *et al.*, 1997). The many combinations of bacteria and yeasts that can produce a lactic and alcoholic fermentation necessary for Kepi production, further increases the variety found in the microbial species of the grains (Duitschaever, 1989). The bacteria isolated from Kepi grains include mainly lactic acid bacteria (LAB) from the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* and acetic acid bacteria (Angulo *et al.*, 1993; Pintado *et al.*, 1996; Rea *et al.*, 1996). Yeasts isolated from Kepi grains include lactose and non-lactose fermenting yeasts from the genera *Saccharomyces*, *Candida*, *Kluyveromyces*, *Torulospora*, *Pichia* and *Cryptococcus* (Angulo *et al.*, 1993; Kaufmann, 1997; Özer & Özer, 1999). However, the presence of some of these microbes have been ascribed to the lack of asepsis during the production of Kepi, as well as contamination from the surrounding environment (Angulo *et al.*, 1993).

Since there are so many factors that can influence the microbial community of Kepi grains, it is very difficult to determine the exact microbial population of the grains. In this study the impact of various factors, including the different stages of Kepi production, grain origin, lyophilisation and packaging in three different packaging materials, on the microbial community of Kepi grains were determined. The microbial enrichment of Kepi grains with propionibacteria and the use of a polymerase chain reaction (PCR) technique to detect propionibacteria in the Kepi beverage and grains, were also evaluated.

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CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

Whole and skim milk are classified as excellent sources of calcium, phosphorus and riboflavin and are also a good source of proteins, thiamine and vitamin A (McGraw & Hill, 1987). In fact, milk is so nutritious it is often stated that milk is the most near perfect food (McGraw & Hill, 1987). However, the main disadvantage of milk as a major food, is that it is perishable and must be stored at 7°C or lower immediately after purchase (Bennion, 1995). This is not always possible in regions with a poorly developed technological and economic environment. In these regions, the warm climate, poor quality of roads and local traditions in the handling of milk, often leads to the natural production of fermented milk products (Bachmann, 1987; Buttriss, 1997).

Fermented milks usually have a nutrient composition that is very similar to that of milk (Steinkraus, 1996). Concentrations of vitamins (with the exception of folic acid) are generally lower in fermented milks, but concentrations of lactic acid, galactose, free amino and fatty acids are increased as a result of the fermentation (Gurr, 1987). It is, therefore, evident that fermentation preserves most of these high quality nutrients present in milk and helps to provide a larger variety of nutritious foods.

It is believed that fermented milks originated in third world countries, however, a wide range of fermented milks ("cultured milks") with different flavours, textures, consistencies and functions are commercially produced world-wide (Bachmann, 1984; Roginski, 1988; Buttriss, 1997). Today, the majority of fermented milk products are made from cows milk, but in certain countries sheep, goat, buffalo and even horse milk are also used in large quantities (Roginski, 1988).

Fermentation of milk is accomplished by the lowering of the pH value (accumulation of lactic acid), the partial depletion of lactose by microbes as an energy and carbon source, the removal of a portion of the moisture of milk which results in lowering the water activity and the production of certain metabolites,

antibiotics and other peptide factors (Vedamuthu, 1982). It is the lactic acid that is responsible for this ideal conservation method of milk, since it spoils the conditions for the development of many pathogens and also exhibits bacteriostatic and even bacteriocidal properties (Bachmann, 1984). The lactic acid fermentation thus enables foodstuffs to be preserved without artificial cooling. A distinctive feature of some fermented milk products is that, in addition to lactic acid fermentation, an alcoholic yeast fermentation also takes place (Koroleva, 1988).

Fermented milk products have a wide range of advantages (Roginski, 1988). They are palatable, widely available at affordable prices and very versatile. In addition to the purely nutritional properties of fermented milk products, there is also increasing support for a number of health and other advantages (Blanc, 1984; Buttriss, 1997; Gates, 1996; Mann, 1989; Shah, 1993). The evidence for some of these health properties, however, still requires convincing scientific proof (Buttriss, 1997; Roginski, 1988; Steinkraus, 1996).

B. THE DEVELOPMENT OF KEPI

Kepi is an ancient, self-carbonated, fermented milk beverage that has its origin in the Russian village of Karatschajeff, at the foot of Elbur in the Caucasus – a 750-mile-mountain range between the Caspian and Black seas (Beuchat, 1978; Duitschaever, 1989; Kurmann *et al.*, 1992; Kwak *et al.*, 1996; Liu & Moon, 1983; Mann, 1979; Saloff-Coste, 1999; Vedamuthu, 1982). Originally the manufacture of Kepi was only known to members of the Ossete and Karabbiner tribes, who had in their possession the so-called Kepi grains that were essential for the manufacture of Kepi (Duitschaever, 1989; Kurmann *et al.*, 1992). In the Orient these grains are sometimes called "Millet of the Prophet" in reference to Mohammed. The name Kepi is derived from the Turkish word keif, denoting pleasant taste or good feeling (Gates, 1996; Kaufmann, 1997; Kurmann *et al.*, 1992). Kepi is also manufactured under a variety of other names, including kephir, kiaphur, kefer, knapon, kefir and kippi (Kwak *et al.*, 1996).

Kepi has been prepared for centuries by stirring milk stored in bags made from the hides of animals (mainly goats) (Kaufmann, 1997; Kurmann *et al.*, 1992). During the day the bags were subjected to sunlight and during the night they were taken into the house and hung near the door (Duitschaever, 1989; Koroleva,

1988). Everyone who entered or left the house had to push the sack with their foot in order to mix the liquid (Koroleva, 1988). The natural occurring organisms fermented the milk, whilst fresh milk was continuously added after some of the fermented milk was removed, thus providing for a continuous natural fermentation (Duitschaever, 1989; Kurmann *et al.*, 1992; Rea *et al.*, 1996). Clay pots, wooden buckets and sheep-skin bags were also used to make Kepi and it has been reported in the literature that during the continuous use of the same containers, their walls became covered with colonies of microbes that resembled boiled rice (Roginski, 1988).

Kepi is commonly manufactured by fermenting milk with a complex mixture of microbes, including various species of lactobacilli, lactococci, leuconostocs and acetic acid bacteria and yeasts, both lactose- and non-lactose-fermenting (Kwak *et al.*, 1996; Saloff-Coste, 1999). It can be made from any type of milk, including cow, goat, sheep, camel, buffalo, soy, coconut and rice (Duitschaever, 1989; Gates, 1996; Pidoux *et al.*, 1990; Steinkraus, 1996) and has the following general characteristics: an acidic pH of at least 4.0; a 0.5 to 2% alcohol content; a 0.8% lactic acid content and a variable fat content that depends on the type of milk that is used (Duitschaever, 1989; Liu & Moon, 1983; Kneifel & Mayer, 1991). Depending on the type of milk used for fermentation, the concentration of certain vitamins (thiamine, pyridoxine and folic acid) are increased, while the concentration of others (orotic acid) are decreased (Kneifel & Mayer, 1991). Other minor components include CO_2, traces of acetaldehyde, diacetyl and acetone (Vedamuthu, 1982). Kepi has a yeasty-sour taste, with a tangy effervescence that is dependent on the composition of the Kepi grain. Kepi milk is known to have a compact and uniform curd and a creamy consistency (Wood, 1998). The slight fizziness and low alcohol content is responsible for the nickname given to Kepi – "The champagne of cultured dairy products" (Merin & Rosenthal, 1986).

It is probable that under different circumstances, different microbes are responsible for the production of Kepi (Duitschaever, 1989), since many combinations of bacteria and yeasts will produce a lactic and alcoholic fermentation. The Kepi flavour, however, is governed by the particular combinations of microbial strains that are embedded in the grains. Other properties that may contribute to the quality of Kepi are the chemical composition,

rheology and organoleptic features (Garrote *et al.*, 1997). The shelf-life of Kepi is about 36 hours, but in glass bottles the product can be kept for 8 – 10 days at 3°-4°C (Koroleva, 1988).

Kepi has been produced on an industrial scale since the early 1930's. It was only at the end of the 1950's, however, that specialists at the All-Union Dairy Research Institute (VNIMI) developed a method for the manufacturing of Kepi, which provided a product with properties close to those of traditional Kepi (Koroleva, 1988). The production of a stirred type Kepi by this method, e.g. fermentation, coagulum formation, agitation, ripening and cooling, takes place in one large vessel equipped with a cooling jacket. Kepi produced by this method is a fermented drink with a typical refreshing, slightly sharp taste and aroma, an alcohol content that is minimal and a mild acidity (Koroleva, 1988). Further attempts to manufacture Kepi commercially have proved to be difficult on account of the problems involved in reproducing the microbes found in the Kepi grain (Duitschaever, 1989; La Rivière *et al.*, 1967; Mann, 1985; Rea *et al.*, 1996).

International interest in the commercial production of Kepi has increased in recent years because of the wide range of advantages of the product. Subsequently, Kepi has become a very popular product in Russia, western and eastern European countries, Poland, Scandinavia, Finland, Sweden, Brazil, Israel, the Middle East and Central Asia (Duitschaever, 1989; Gates, 1996; Koroleva, 1988; Kwak *et al.*, 1996; La Rivière *et al.*, 1967; Mann, 1983; Rašić, 1986; Roginski, 1988; Saloff-Coste, 1999; Wood, 1998). In these countries Kepi is consumed with meals or as a snack before bedtime and can be sweetened with sugar or combined with fruits or biscuits (Steinkraus, 1996).

C. KEPI GRAINS

Characteristics

Kepi grains are small, irregularly shaped and their colour varies from a gelatinous white or cream to yellow (Kwak *et al.*, 1996; Liu & Moon, 1983; Pintado *et al.*, 1996; Roginski, 1988; Steinkraus, 1996; Vedamuthu, 1982). The grains are tough and resilient (Abraham & De Antoni, 1999) and look like the individual florets of a cauliflower, varying in size from roughly 0.5 to 3.0 cm (Garrote *et al.*, 1997;

Kwak *et al.*, 1996; Liu & Moon, 1983; Merin & Rosenthal, 1986; Rea *et al.*, 1996; Roginski, 1988; Vedamuthu, 1982; Wood, 1998).

The grains are composed of proteins and polysaccharides in which the microbes are embedded (Garrote *et al.*, 1997). The lactic acid bacteria and yeasts are found within the folds of the grains, as colonies of symbiotic microbes (Garrote *et al.*, 1997; Liu & Moon, 1983; Saloff-Coste, 1999; Vedamuthu, 1982). Electron microscope photographs have revealed a cobweb-like network holding the yeasts and bacteria together at the centre of the grains (Mann, 1985).

Kepi grains develop under specific physical and chemical conditions that inhibit the development of most other unwanted microbes (Angulo *et al.*, 1993). The exact number of microbes in the grains cannot easily be determined due to the heterogeneous distribution of the microbes on the surface and the difficulty in separating the microbes from the polysaccharide matrix (Garrote *et al.*, 1997). It is known, however, that only part of the microbial composition of the grains is specific (La Rivière *et al.*, 1967) and, furthermore, that the number and position of each specific microbe is not constant. This may be explained by the different origins of the Kepi grains, the different methods of their cultivation and the storage conditions (Garrote *et al.*, 1997; Pintado *et al.*, 1996; Rašić, 1986). However, the balance of microbes in the grains are stable and specific (Garrote *et al.*, 1998).

Rea *et al.* (1996) examined the interior and the exterior of Kepi grains with the electron microscope and found considerable variation between the different areas of the grains (Mann, 1989). It has been reported that whereas the peripheral part of the grain is populated almost exclusively by bacteria, the centre of the grain is dominated by yeasts (Bottazzi & Bianchi, 1980; Lin *et al.*, 1999). The intermediate areas contain a balance between bacteria and yeasts, which changes progressively according to the distance from the core (Özer & Özer, 1999). The microbial make-up of Kepi grains is, however, hard to define. Wood (1998) reported that lactobacilli (homo- and heterofermentative, meso- or thermophilic) makes up about 65 - 80% of the microbial content, while lactococci and some lactose and non-lactose-fermenting yeasts makes up the remaining 20%. Angulo *et al.* (1993), however, studied eight Kepi grains from individual dairies in various parts of the North-West of Spain and found lactic acid bacteria (LAB) to make up between 30 – 60% of the grains, while yeasts made up 33.3 – 60% of the grains.

The contaminant concentration of the grains were reported to be between 6.6 – 33.3%.

The grains are insoluble in water and common solvents and become slimy or jelly-like when they are soaked (Vedamuthu, 1982; Wood, 1998). In milk, the grains swell and turn white. As the grains are allowed to grow, microbes are shed from the grains into the milk. This is made possible by the structure of the Kepi grains which is more even at the edges making it easier for the microbes to escape from the grain into the surrounding milk (Mann, 1985). In the milk the microbes continue to multiply (Garrote *et al.*, 1998; Liu & Moon, 1983; Kurmann *et al.*, 1992). A Kepi beverage of high quality has the following microbial composition: 10^7 - 10^8 thermophilic lactobacilli; 10^9 homofermentative mesophilic lactic acid streptococci; 10^7 - 10^8 heterofermentative lactic acid streptococci; 10^4 - 10^5 yeasts; and 10^4 - 10^5 acetic acid bacteria (Koroleva, 1988).

Mann (1985) found that the total solid content of the Kepi grains is about 10% (m/v) and this is composed of 32.6% protein, 56.2% carbohydrates, 3.5% fat and 6% ash. According to Garrote *et al.* (1997), the chemical composition of Kepi grains is ($g.kg^{-1}$): 890 - 900 water, 60 sugars, 30 protein, 2 lipid and 7 ash. The composition of the Kepi grains depends on many factors, including the type of milk used as substrate, the type and size of the grains and the technological conditions used during Kepi manufacturing (Wood, 1998).

The activity of the grains depends on the viability of the microbes and some difficulties have been experienced in maintaining good quality Kepi grains to produce a Kepi with the appropriate and acceptable viscosity (Garrote *et al.*, 1997). Research has shown that, whereas grains stored in water can only be kept for 8 - 10 days, dried Kepi grains can be kept for 12 - 18 months (Garrote *et al.*, 1997; Vedamuthu, 1982). It has also been reported that the preservation of Kepi grains at –80°C changes the microbial composition of the grains less than preservation of the grains at –20° or –4°C (Garrote *et al.*, 1997). In addition, milk fermented with grains frozen at –80°C have the same viscosity, level of acidity and carbon dioxide content as milk fermented with control grains, whereas milk fermented with grains stored at 4°C changes the microbial composition and produce a product with unpleasant characteristics (Garrote *et al.*, 1997). Kepi grains can also be freeze-dried (Brewer, 1998).

Kefiran

Kefiran is a matrix of fibrillar amorphous material that consists largely of polysaccharides and forms about 24 - 25% of the dry weight of the Kepi grains (Duitschaever, 1989; Kooiman, 1968; Mann, 1989; Merin & Rosenthal, 1986; Pintado *et al.*, 1996; Saloff-Coste, 1999). The kefiran holds the microbes of the grains together (Roginski, 1988). It is believed that this polymer is produced by the predominating bacterial species (Steinkraus, 1996) which separates non-carbohydrate producing populations of lactobacilli from yeasts so that sheet-like structures show asymmetry. The yeasts predominates on the one side with the reverse almost completely populated with lactobacilli (Marshall *et al.*, 1984a).

Kefiran is synthesized together with new cells and consists of equal parts of D-galactose and D-glucose (Kooiman, 1968; Pintado *et al.*, 1996; Roginski, 1988; Steinkraus, 1996). Mukai *et al.* (1988) showed that apart from glucose and galactose, kefiran also contains a repeating unit of 6-0-substituted galactose. Milk fat and denatured milk protein is also associated with this polysaccharide matrix (Kurmann *et al.*, 1992). Rheological studies have shown that kefiran has a very low viscosity in solution and is unable to form rigid gels in the absence of ethanol (Pintado *et al.*, 1996).

Several homofermentative *Lactobacillus* species, including *L. kefir* and *L. kefiranofaciens*, produce this polysaccharide, but it is still not clear which *Lactobacillus* is the main polysaccharide producer (Kooiman, 1968; La Rivière *et al.*, 1967; Roginski, 1988; Rosi & Rossi, 1978; Steinkraus, 1996). Many researchers, however, do not believe *L. kefir* to be the main polysaccharide producing bacterium (Kandler & Kunath, 1983; Liu & Moon, 1983), due to the rapid loss of its capacity to form kefiran capsules during isolation from the Kepi grain (La Rivière *et al.*, 1967; Liu & Moon, 1983).

Toba *et al.* (1986), Fujisawa *et al.* (1988) and Rea *et al.* (1996) identified *Lactobacillus kefiranofaciens* as the main polysaccharide producer. It was found, however, that *L. kefiranofaciens* requires wine in the growth medium for the production of polysaccharides and then only produces a low yield of polysaccharides (Yokoi *et al.*, 1991). Other lactic acid bacteria, e.g. *Leuconostoc mesenteroides* and *Lactococcus lactis* subsp. *cremoris* were also found capable of producing extracellular polysaccharides (Marshall *et al.*, 1984a).

There are some restrictions on the commercial use of kefiran as a food thickening or gelling agent, however, there might be some useful applications as a food additive (Pintado *et al.*, 1996). Kefiran, furthermore, exhibits antitumor activity on Ehrlich carcinoma and Sarcoma 180 solid tumor (Shiomi *et al.*, 1982) and anti-metastatic activity against Lewis lung carcinoma and the highly metastatic B16 melanoma in mice (Furukava, 2001). This possible health aspect might emphasize the importance of identifying the polysaccharide-producing bacteria correctly and finding a procedure for the mass production of kefiran (Yokoi *et al.*, 1990).

D. MICROBIOLOGICAL COMPOSITION OF KEPI GRAINS

Lactobacillus

Lactobacilli are members of the lactic acid bacteria (LAB) group, which traditionally includes the genera *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus*. In practice, however, the LAB group is often expanded to include a wide variety of other microbes (Arihara & Luchansky, 1995). Lactobacilli are Gram-positive, non-spore forming, microaerophilic rods and usually non-motile (Hammes *et al.*, 1992; Arihara & Luchansky, 1995; Roginski, 1988; Kandler & Kunath, 1983). These bacteria ferment hexoses and pentoses or just hexoses and they can be either homofermentative, producing more than 85% lactic acid, or heterofermentative, producing lactic acid, carbon dioxide, ethanol and/or acetic acid in equimolar amounts (Hammes *et al.*, 1992; Roginski, 1988).

The taxonomic position of the lactic acid rods dominating in Kepi grains and Kepi is still uncertain (Kandler & Kunath, 1983). Marshall *et al.* (1984b) indicated that "*Lactobacillus caucasicus*" was most frequently found to be part of Kepi grains. This organism was first observed by Beijerinck in 1889, but an authentic strain of this species no longer exists. The epithet "*caucasicus*" is no longer recognised, since the type culture deposited at the American Type Culture Collection (ATCC) was found to be a mixture of different lactobacilli (Marshall *et al.*, 1984a). These Kepi isolates were subsequently named "*Lactobacillus desidiosus*", while La Riviére *et al.* (1967), Pederson (1938) and Rosi & Rossi (1978) described them as strains of *Lactobacillus brevis.* Kandler & Kunath (1983), however, reinvestigated the *Lactobacillus* species and re-named them

Lactobacillus kefir. The isolates showed 85 - 90% homology with "*Lactobacillus caucasicus*". *Lactobacillus kefir* seems to predominate over the rest of the LAB in the Kepi grains (Kandler & Kunath, 1983; Kooiman, 1968; Kunath, 1983; La Riviére *et al.*, 1967). It constitutes roughly 80% (m/v) of the lactobacilli in the Kepi beverage and about 10% (m/v) in the grain, whereas the other lactobacilli predominate in the grain (90%) and represents only 20% of the lactobacilli in the beverage (Saloff-Coste, 1999).

Bottazzi & Bianchi (1980) suggested that the lactobacilli predominate at the edge of the Kepi grain. The predominance of the species, however, depends on the origin of the Kepi grains (Angulo *et al.*, 1993). Grains from different origins (Table 1) revealed some similarity in the specific species that were found, but showed that the combinations of these species may vary. The lactobacilli most frequently found in the grains, except for *Lactobacillus kefir,* are the falcultative heterofermentative, *Lactobacillus casei* subsp. *rhamnosus* and the obligate homofermentative, *Lactobacillus dulbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus* species (Koroleva, 1988). Further studies on the lactobacilli in Kepi grains also revealed the presence of *Lactobacillus kefiranofaciens* (Fujisawa *et al.*, 1988), the homofermentative, *Lactobacillus kefirgranum* and the heterofermentative, *Lactobacillus parakefir* (Takizawa *et al.*, 1994).

Of the lactobacilli found in the Kepi grains, the curved lactobacilli appeared to be autolysing (Marshall *et al.*, 1984a; Sobczak & Kocon, 1982) with a viability of only 3% (La Riviére *et al.*, 1967). It was speculated that the loss of viability could possibly be due to the insoluble matrix in which these lactobacilli are embedded, in that it may impose a diffusion barrier to nutrients (Marshall *et al.*, 1984a). Furthermore, it appears as if the distribution of lactobacilli in the Kepi grains and in the Kepi culture does not differ extensively. Garrote *et al.* (1997) and Marshall (1993) found 10^9 cfu.g^{-1} lactobacilli in the grains and 10^9 – 10^{10} cfu.ml^{-1} and 10^8 cfu.ml^{-1} lactobacilli respectively in the Kepi beverage.

Lactococcus

The genus *Lactococcus* is composed of Gram-positive, non-spore forming cocci that can be facultatively or strictly anaerobic. They are homofermentative

Table 1. Lactobacilli content of Kepi grains from different origins.

Origin	***Lactobacillus* species**
Taiwan (Lin *et al.*, 1999)	*Lb. helveticus*
Poland, Norway (Mann, 1985)	*Lb. casei*
Denmark (Iwasawa *et al.*, 1981)	*Lb. kefir*, *Lb. buchneri*
Russia, Bulgaria, Yugoslavia (Liu & Moon, 1983)	*Lb. kefir*, *Lb. acidophilus*
Galican region (Angulo *et al.*, 1993)	*Lb. acidophilus*, *Lb. gasseri*, *Lb. casei* subsp. *rhamnosus*, *Lb. casei* subsp. *pseudoplantarum*, *Lb. casei* subsp. *tolerans*, *Lb. fermentum*, *Lb. kefir*, *Lb. viridiscens*

bacteria that produce lactic acid from glucose and some species including *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* will also produce diacetyl, a major aroma-contributing substance, and carbon dioxide from nitrate (Singleton, 1995). These bacteria are employed in single and mixed cultures for the production of different kinds of cheese, fermented milks, cultured butter and casein (Singleton, 1995).

Small numbers of lactococci have been isolated from Kepi grains, but numbers of 10^9 cfu.ml^{-1} lactococci (Marshall, 1993) were found to be present in the Kepi beverage (Bottazzi & Bianchi, 1980; Duitschaever *et al.*, 1988; Garrote *et al.*, 1997; Kandler & Kunath, 1983; Marshall *et al.*, 1984a; Rea *et al.*, 1996). This suggests that the lactococci are only lightly bound to the Kepi grain structures during growth and are easily washed off the grains during sample preparation or shed into the milk during fermentation. The pH of the interior of the Kepi grain is also very low due to the high acid-producing activity of the lactobacilli and this inhibits the growth of the lactococci (Rea *et al.*, 1996; Saloff-Coste, 1999).

Many of the *Lactococcus* species found in Kepi grains were previously included in the genus *Streptococcus*. This genus, however, was subjected to some taxonomic changes and is now divided into the genera *Streptococcus*, *Enterococcus* and *Lactococcus* (Ruoff, 1992). *Lactococcus lactis, Lactococcus lactis* subsp. *cremoris* and *Lactococcus durans* are the lactococci most commonly found in Kepi grains (Keller & Jordaan, 1990; Koroleva, 1988; Kunath, 1983; Libudzisz & Piatkiewicz, 1990). Other lactococci species isolated from Kepi grains include *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *diacetylactis* (Angulo *et al.*, 1993; Garrote *et al.*, 1997; Marshall, 1993; Rosi & Rossi, 1978). Grains studied by Kwak *et al.* (1996) also contained the species *Lactococcus filant*, though the presence of this specific species has not been reported elsewhere.

Leuconostoc

The genus *Leuconostoc* comprises nine species that are Gram-positive, non-motile, non-spore forming and are facultative anaerobes (Holzapfel & Schillinger, 1992; Singleton, 1995). This group of bacteria ferments carbohydrates to carbon dioxide, ethanol and lactate, although some strains may form acetate instead of ethanol. It was found that although ethanol is in most cases an end-

product of sugar metabolism by leuconostocs, the co-metabolism of lactose and citrate by leuconostocs results in no ethanol production. These citrate utilizing bacteria also produce diacetyl, the main aroma-forming substance in Kepi, in amounts of about 1 $mg.l^{-1}$ (Wood, 1998).

Except for *Leuconostoc lactis*, species of this genus do not usually grow in milk, but their growth is stimulated by lactococci (Boquien *et al.*, 1988) and by the addition of yeast extract to the milk, as well as, in the case of Kepi, by the growth of yeasts themselves (Rea *et al.*, 1996). The growth of leuconostocs are, however, desirable in dairy fermentation for their production of antibiotics and their relative resistance to bacteriophage attack (Levata-Jovanoric & Standine, 1997). Samples isolated from Kepi grains from Russia, Bulgaria and Yugoslavia showed the presence of *Leuconostoc kefir* (Liu & Moon, 1983), while samples isolated from grains from Taiwan showed the presence of *Leuconostoc mesenteroides* (Lin *et al.*, 1999). *Leuconostoc mesenteroides* subsp. *dextranicum* and *Leuconostoc mesenteroides* subsp. *cremoris* were also isolated from Kepi grains (Koroleva, 1988; Kwak *et al.*, 1996).

Acetic Acid Bacteria

The acetic acid bacteria include the genera *Acetobacter* and *Gluconobacter* (Swings, 1992). *Acetobacter* has peritrichous flagella and oxidises acetic acid and lactic acid to carbon dioxide and H_2O, whereas *Gluconobacter* has polar multitrichous flagella and do not oxidise acetic and lactic acid to carbon dioxide. In addition, *Acetobacter* prefer ethanol or lactate to glucose for growth, while *Gluconobacter* prefer glucose to ethanol for growth (Singleton, 1995). Both genera comprises Gram-negative rods that are non-spore forming, strictly aerobic and either motile or non-motile (Singleton, 1995; Swings, 1992).

Being strictly aerobic, the presence of acetic acid bacteria in what is essentially an anaerobic fermentation is actually highly unusual (Rea *et al.*, 1996). Kepi grains have been found to contain *Acetobacter aceti* and *Acetobacter rancens* (Koroleva, 1988, Saloff-Coste, 1999), which are thought to play an important role in maintaining the microbiological symbiosis of the Kepi grains and improve the Kepi consistency by increasing its viscosity (Saloff-Coste, 1999, Wood, 1998). The acidification activity of lactococci also increases when they are cultivated together with these acetic acid bacteria (Keller & Jordaan, 1990).

Acetic acid bacteria were present in the fermented milk of four of the six Kepi grains studied by Rea *et al.* (1996), reaching levels of ~10^5 cfu.ml^{-1} at the end of the fermentation. In their studies the cessation of growth after ~20 hours was attributed to the depletion of oxygen. Levels of *Acetobacter* sp. of $10^3 - 10^7$ cfu.ml^{-1} was also reported by Koroleva (1988). These microbes are considered to be contaminants by some workers (Angulo *et al.*, 1993), however, they produce vitamin B_{12} and may therefore stimulate the growth of other organisms in the grains (Zourari & Anifantakis, 1988).

Pediococcus

The genus *Pediococcus* currently includes eight species: *damnosus*, *parvulus*, *inopinatus*, *dextrinicus*, *pentosaceus*, *acidilactici*, *urinaeequi* and *halophilus* (Ray, 1995). The cells are spherical and form tetrads, but can also be present in pairs. They are non-motile, non-spore forming and facultatively anaerobic. Pediococci ferment glucose to lactate, but the carbohydrate fermentation pattern differs between the species in the genus (Ray, 1995).

Angulo *et al.* (1993) found *Pediococcus* species in only one sample of Kepi grains from the Galician region and considered this genus to be a contaminant. It appears that the presence of pediococci depends on the different microbiological qualities of the milks used and the lack of asepsis during the manufacturing of Kepi (Angulo *et al.*, 1993).

Propionic Acid Bacteria

The genus *Propionibacterium* is a member of the *Actinomycetaceae* group (Lyon & Glatz, 1995). *Propionibacterium* species were first described in 1909 by Orla-Jensen and the genus has been divided into two main groups: the cutaneous propionibacteria and the classical or dairy propionibacteria (Lyon & Glatz, 1995). They are Gram-positive and the cells can be either branched or unbranched rods or coccoid forms (Singleton, 1995). These bacteria are also non-motile and anaerobic. The classical propionibacteria are important starter organisms in dairy fermentations, may contribute to natural fermentations of silage and olives and can produce a variety of industrially important products (Lyon & Glatz, 1995). Somkuti & Johnson (1990) even reported cholesterol uptake by strains of *freudenreichii*. Currently, five species of dairy propionibacteria are recognised: *freudenreichii*

subsp. *freudenreichii*, *freudenreichii* subsp. *shermanii*, *thoenii*, *acidipropionici* and *jensenii*.

The propionibacteria ferment the lactic acid produced by the starter lactobacilli from lactose, to propionic and acetic acids and carbon dioxide (Lyon & Glatz, 1995). The organic acids do not only contribute to flavour, but are also inhibitory to certain moulds, yeasts and bacteria. In addition, the produced organic acids are known to improve the shelf-life of fermented products like Kepi (Lyon & Glatz, 1995; Somkuti & Johnson, 1990).

The importance of propionibacteria in Kepi, however, is its capability to produce vitamin B_{12} (Cobalamin) (Lyon & Glatz, 1995). The increasing demand for more nutritional and functional foods has emphasised the importance of knowing more about the changes that take place in the vitamin B_{12} content during fermentation (Alm, 1982). Vitamin B_{12} is the only vitamin that contains essential mineral elements. It cannot be made synthetically and must be grown in bacteria or moulds (Gates, 1996). In the body, this vitamin has many important functions. It is an important cofactor for the metabolism of carbohydrates, lipids, amino acids and nucleic acids (Quesada-Chanto *et al.*, 1994). It helps accomplish the synthesis of RNA and DNA and is necessary for the normal metabolism of nerve tissue and for red blood cell formation. Vitamin B_{12} also helps build immunity and facilitates the synthesis of choline and porphyrin (Gates, 1996; Marshall, 1986). A deficiency of vitamin B_{12} prevents optimum red blood cell development, especially in children, and leads to anaemia (Marshall, 1986).

Studies determining the influence of the growth of lactic acid bacteria on the level of vitamin B_{12} have shown that the concentration of this vitamin decreased between 15 and 95% during lactic acid fermentation of milk (Liu & Moon, 1983). The addition of propionibacteria to Kepi, however, results in increases, or only small losses, of vitamin B_{12}. Kepi produced using Kepi grains together with "*Propionibacterium freudenreichii* subsp. *shermanii* " was found to be a product with a high food value, rich in proteins and vitamins, including vitamin B_{12} (Liu & Moon, 1983). A method for the vitamin B_{12} enrichment of Kepi comprised inoculating 5% of the "*shermanii*" culture together with 1 – 5% of ordinary Kepi culture, into the Kepi milk. The vitamin B_{12} content of the Kepi was 14.3 $\mu g.100ml^{-1}$, compared to 0.51 μg in control Kepi not containing "*shermanii*", representing a

28 fold increase (Mann, 1979). A 60 fold increase in vitamin B_{12} levels has also been achieved (Černá *et al.*, 1977).

The addition of "*shermanii*" is claimed to have no adverse effect on the organoleptic properties of Kepi (Černá & Hrabová, 1977; Mann, 1979), but altering the ratio of microbes in addition to including "*shermanii*" in Kepi may affect the fermentation pattern (Liu & Moon, 1983). The growth of bacteria and yeasts during the fermentation process for instance, was found to be slightly inhibited by "*shermanii*". Polish researchers also reported successful vitamin B_{12} enrichment of Kepi using a culture of "*petersonii*" T-112 in combination with Kepi grains (Mann, 1979).

Yeasts

Yeasts are unicellular, spore forming, non-motile, aerobic microbes that reproduces either asexually by budding and transverse division or sexually through spore formation (Prescott *et al.*, 1996). Generally, the spherical to egg shaped yeast cells are larger than bacteria and they may also vary considerably in size between species (Yadav & Mishra, 1995).

All of the yeast strains are derived as "contaminants" from the environment, since the milk environment and its products allow growth of a vast range of yeasts. The yeasts, that survive under dairy type selective pressures exerted by the internal and external environments of Kepi, are responsible for promoting symbiosis among the microbes, carbon dioxide formation and the development of part of the characteristic taste and aroma of Kepi, with a low production of alcohol (Koroleva, 1988; Kwak *et al.*, 1996). Specific yeasts, that are capable of fermenting lactose and other sugars, may continue fermentation even after the product is packaged, provided that the temperature is favourable (Koroleva, 1988).

Lactose fermenting yeasts have been reported to be present in the peripheral layers of the Kepi grains, with the non-lactose fermenting yeasts in the inner layers (Mann, 1989; Wood, 1998). There is also an emergence of higher numbers of yeasts in the median, with the centre of the grain composed almost entirely of yeast species set in a matrix (Kunath, 1983; Rea *et al.*, 1996). Garrote *et al.* (1997) and Wood (1998) found approximately 10^8 cfu.g^{-1} yeasts in the Kepi grains, while 10^6 - 10^7 cfu.ml^{-1} yeasts were present in the Kepi culture.

Predominant yeasts that have been isolated from Kepi grains, include *Torulopsis holmii* and *Saccharomyces delbrueckii* in a ratio of about 10:1, both of which are unable to ferment lactose (Steinkraus, 1996). Marshall *et al.*(1984b), however, found that the two most commonly isolated Kepi yeasts are *Candida kefir* and *Sacch. cerevisiae*, while Pintado *et al.* (1996) described *Sacch. delbrueckii* as the yeast most frequently isolated from Kepi grains. Other yeasts found in Kepi grains include *Sacch. Carlsbergensis*, *Sacch. Globosus*, *Sacch. Florentinus*, *Sacch. kefir Sacch. Lactis*, *Kluyveromyces marxianus* subsp. *marxianus*, *Kluyv. marxianus* subsp. *bulgaricus*, *Kluyv. Fragilis*, *Kluyv. Lactis*, *Torulospora delbrueckii*, *Torula kefir*, *Candida* Pseudotropicalis, *C. tenuis* and *Cryptococcus kefyr* (Kaufmann, 1997; Koroleva, 1988; Kwak *et al.*, 1996; Liu & Moon, 1983). *Sacch. Lipolytic*, *Brettanomyces anomalus*, *C. Holmii*, *C. Valida*, *C. friedricchii* and *Pichia fermentans* have also been isolated from Kepi grains (Garrote *et al.*, 1997; Lin *et al.*, 1999; Mann, 1979).

The Kepi beverage usually contains yeasts numbers that range from less than 100 to 10 000 yeasts.ml^{-1} (Garrote *et al.*, 1997; Mann, 1989; Wood, 1998). In many cases, the yeasty flavour normally associated with a quality Kepi was found to be absent even in samples containing more than 100 000 yeasts.ml^{-1}. The flavour, therefore, appears to be dependent on the yeast species and not the number of yeasts present.

In the literature it has also been reported that a certain mould or mycelial fungus has also repeatedly been isolated from Kepi grains and identified as *Geotrichum candidum* (Garrote *et al.*, 1997; Pintado *et al.*, 1996; Roginsky, 1988). *Geotrichum candidum* usually covers the surfaces of the Kepi grains if present, but was found not to affect the performance of the grains or influence the organoleptic properties of Kepi (Roginsky, 1988).

E. CONCLUSION

Kepi grains, as well as the Kepi beverage, consist of a microbial population that varies greatly. This microbial population is dominated by lactobacilli and yeasts, which suggests that these microbes play an integral part in the fermentation of milk resulting in the production of Kepi. It is clear, however, that it is the combination of microbes, rather than the specific microbes present in the grain,

that results in a good quality Kepi beverage. Factors such as the origin of the Kepi grains and the way the grains are handled, also help to determine the microbial population of the grains. Although many different studies have been done on Kepi grains, it is only the lactic acid bacteria that is well understood. Uncertainty exist about whether either pediococci or acetobacter form an integral part of all or some Kepi grains or whether these two species are merely contaminants. Little is also known about the presence of propionibacteria in any Kepi grains even though the Kepi grain environment is perfect for the growth of these bacteria. It is therefore necessary to take a more detailed look at the microbial population of Kepi grains, as well as the factors influencing it.

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CHAPTER 3

ISOLATION AND CHARACTERISATION OF THE MICROBIAL POPULATION OF KEPI GRAINS FROM VARIOUS SOURCES

Abstract

Kepi is a slightly acidic fermented milk beverage that is produced by adding lactic acid bacteria and yeasts in the form of grains, to milk. The bacteria and yeasts present in the Kepi grains are known to vary widely as the population of the grains are influenced by factors such as the production method, source of the grains and the age of the grains. In this study the impact of different stages in the production of Kepi and Kepi grains, as well as the impact of grain source were investigated. Selective growth media and morphological and biochemical characteristics were used for the isolation and identification of the microbes present in the Kepi grains. Based on the data obtained there was a decrease in the microbial numbers obtained from locally produced Kepi grains after three days of activation (1.1×10^8 cfu.g^{-1}), 10 d of mass production (6.2×10^7 cfu.g^{-1}) and 30 d of Kepi production (8.3×10^6 cfu.g^{-1}). The microbial numbers from Kepi grains obtained from eight different sources varied between 1.7×10^8 cfu.g^{-1} and 6.0×10^5 cfu.g^{-1}.

There was a definite variation in the microbial population isolated from locally produced Kepi grains at the various stages of Kepi and Kepi grain production. The addition of yeast extract and urea and the stress conditions applied during the mass production of the grains resulted in the development of *Candida lambica* and *C. krusei*. *Lactobacillus plantarum* was also isolated from the mass produced Kepi grains, but not from Kepi grains at any other stage of Kepi production. Furthermore, Kepi grains obtained from different sources also consisted of different microbial combinations, confirming that the origin of Kepi grains influence the microbial composition. None of the Kepi grains, whether locally produced or obtained from other sources, contained any pediococci, acetic acid bacteria or propionibacteria.

Introduction

Kepi differs from other fermented milk products in that it is not the result of the metabolic activities of microbes that are uniformly distributed throughout the body of the milk (Steinkraus, 1996). Fermentation of the milk is accomplished with various microbes grouped together in a cauliflower-like structure (Marshall & Cole, 1985; Toba *et al.*, 1986). At the end of the fermentation the mixed microbial population can be recovered as a solid matrix called the Kepi grain (Rea *et al.*, 1996). The Kepi grains are usually white or yellow, small, irregular and gelatinous (Marshall *et al.*, 1984; Roginski, 1988; Steinkraus, 1996).

The Kepi grains contain a complex mixture of lactic acid bacteria (LAB), yeasts and acetic acid bacteria (Garrote *et al.*, 1997; Saloff-Coste, 1999). A symbiotic relationship exists between the bacteria and the yeasts (Vedamuthu, 1982) and their combined growth results in a fermented product with distinct flavour characteristics and a fizziness due to CO_2 production (Rea *et al.*, 1996). Lactic acid bacteria found in the Kepi grains include homofermentative and heterofermentative *Lactobacillus*, *Lactococcus* and *Leuconostoc* species (Angulo *et al.*, 1993; Pintado *et al.*, 1996; Rea *et al.*, 1996), while the yeasts include members of the genera *Saccharomyces*, *Candida*, *Kluyveromyces*, *Torulopsis* and *Cryptococcus* (Angulo *et al.*, 1993; Iwasawa *et al.*, 1982; Kaufmann, 1997; Özer & Özer, 1999). *Acetobacter rancens* and *A. aceti* are the only acetic acid bacteria that have been isolated from Kepi grains (Koroleva, 1988; Saloff-Coste, 1999). The microbial composition of Kepi grains shows variations depending on the origin of the grains, the cultivation method and the manufacturing method (Pintado *et al.*, 1996). Under different environmental conditions many combinations of bacteria and yeast species will also produce a lactic acid and alcoholic fermentation, thereby producing Kepi (Duitschaever, 1989).

The microbes in the Kepi grains are embedded in a slimy, water-insoluble polysaccharide matrix referred to as kefiran, that is composed of equal amounts of D-glucose and D-galactose (Pintado *et al.*, 1996). It is believed that this polymer is produced by several homofermentative *Lactobacillus* species, including *Lb. kefir* and *Lb. kefiranofaciens* (Rosi & Rossi, 1978). However, it is still not clear which *Lactobacillus* is the main polysaccharide producer (Kooiman, 1968; La Rivière *et al.*, 1967; Roginski, 1988).

The growing popularity of fermented milk products in recent years (Liu & Moon, 1983; Mann, 1989; Oberman & Libudzisz, 1998) has emphasised the importance and need to identify the microbes involved in the production of a product like Kepi. This knowledge will enable the development of procedures that will lead to the production of a milk beverage with characteristics similar to that of traditionally manufactured Kepi. The aim of this study was to isolate and identify the microbial species present in Kepi grains from local and other sources and at different stages of Kepi production, using selective growth media and morphological and biochemical characteristics.

Materials and methods

Starter cultures

The Kepi grains chosen for this study, as well as their original sources are given in Table 1. The grains were obtained from the Department of Food Science, University of Stellenbosch, South Africa, and from Dr J.F. Mostert at the Dairy Research Institute, Pretoria, South Africa. The grains were stored at -18°C before use.

Kepi grain activation and Kepi production

The Kepi grains of local origin (grain type-0) were activated by adding 18 g of the grains to 500 ml of double pasteurised full cream milk, followed by incubation at 25°C. The milk that was used for Kepi production was purchased at the local supermarket and therefore already pasteurised. The milk was, however, pasteurised again (80°C for 20 min) before it was used for Kepi grain activation and the subsequent production of Kepi to ensure that there were no other microbes present in the milk before the grains were added, hence the term "double pasteurised". After 24 h, the grains were sieved out and placed in 500 ml of fresh double pasteurised full cream milk. This step was repeated for three consecutive days.

Kepi was then produced by adding the 18 g of grains to 1 l of double pasteurised full cream milk at 25°C. The grains were sieved out and transferred to 1 l of fresh double pasteurised full cream milk every 24 h. This procedure was repeated until the grains were used to isolate and identify the microbial population at specified time intervals. In the case of the grains from other sources (Table

Table 1. Sources of the Kepi grains used in this study.

Grain Type	Original Source
0 (Local)	Food Science Department, Stellenbosch, South Africa
1	Dairy Research Institute, Irene, South Africa*
2	Local household in South Africa*
3	Local household in South Africa*
4	Local household in South Africa*
5	Local household in South Africa*
6	Local household in South Africa*
7	L. Eksteen, Darleon (from C. Hansen, Denmark)
8	J. Strydom, Seravac

*Obtained from Dr J.F. Mostert, Dairy Research Institute, Irene, South Africa

1), a 2 g sample, which was all that was available, was activated in 20 ml double pasteurised full cream milk at 25°C for 24 h, after which the population was determined.

Mass production of Kepi grains

The mass production of the Kepi grains was done according to the method developed by Schoevers (2000) and patented (SA Patent 2000/1896). The procedure involved the addition of 2% (w/v) yeast extract (Biolab) and 0.5% (w/v) urea (Biolab) to 400 ml of double pasteurised full cream milk. Forty grams of Kepi grains were then added to the pasteurised milk and the containers incubated at 25°C in a shake waterbath. The milk mixture was completely replaced every 24 h.

Isolation of the microbial population

In each case, 10 g of Kepi grains were homogenized in 90 ml (1:10 dilution) of sterile saline solution (0.85% (w/v) NaCl) in a Stomacher (BagMixer, Interscience, France) for 15 min. The same procedure was followed for the Kepi grains from other sources, except that only 2 g grains were homogenised in 18 ml (1:10 dilution) of sterile saline solution.

Microbiological analyses

The concentrations of the viable bacteria and yeasts in the suspensions were obtained by serial plating dilutions in sterile saline solution from 10^{-1} to 10^{-6} on the various selective media, as given in Table 2, and the results were expressed as colony forming units per gram of Kepi grain ($cfu.g^{-1}$). The media that was used for the selection of lactobacilli, lactococci, leuconostocs and propionibacteria were incubated anaerobically using the Anaerocult A system (Merck) for 5 to 10 d at 30°C, after which the selected colonies (Harrigan & McCance, 1976) were cultivated on MRS-medium. The plates with the yeasts and acetic acid bacteria were aerobically incubated for 3 to 5 d at 25°C. The selected colonies from the media that selected for yeasts (MEA and YEC) were cultivated on PDA (Biolab), while colonies from the acetic acid bacteria selective medium (APM) were cultivated on APM. The Harrison Disc method (Harrigan & McCance, 1976) was used to determine the dominant colonies and how many

Table 2. Selective media used for the isolation of the microbial species present in Kepi grains.

Isolation medium [a]	**Selected microbes**
MRS-medium (Biolab) with 3% (w/v) ethanol (Merck) and 0.5% (w/v) filter sterilised cycloheximide (Merck) (pH 6.0) (Pintado *et al.*, 1996).	Lactobacilli (MRS)
KCA-medium ($g.l^{-1}$): tryptone (Biolab) 20.0; yeast extract (Saarchem) 5.0; gelatine (Merck) 2.5; glucose (Merck) 5.0; lactose (Merck) 5.0; sodium chloride (Saarchem) 4.0; tri sodium citrate.$2H_2O$ (Saarchem) 2.0; calcium lactate.$5H_2O$ (Saarchem) 8.0; agar (Biolab) 15.0; calcium citrate (Saarchem) 10.0 and carboxymethyl cellulose (Merck) (1.5% w/v) 100 ml. Ten milliltre filter sterilised TTC (Oxoid) was added (pH 6.6) (Nickels & Leesment, 1964).	Lactococci (KCA+TTC)
KCA-medium with 30 µg filter sterilized vancomycin (Fluka) (pH 6.6) (Benkerroum *et al.*, 1993).	Leuconostocs (KCA+V)
APM-medium ($g.l^{-1}$): malt extract (Biolab) 15.0; yeast extract (Saarchem) 5.0 and agar (Biolab) 15.0. Sixty millilitre filter sterilised ethanol (Merck) (50% v/v) was added (pH 6.8) (DSMZ, 2001).	Acetic acid bacteria (APM)
YELN-medium ($g.l^{-1}$): yeast extract (Biolab) 5.0; sodium lactate (Saarchem) (60% v/v) 20.0; agar (Biolab) 15.0 and Tween 80 (Merck) 1.0 ml. Ten millilitre filter sterilised naladixic acid (0.02%) was added (pH 7.2) (Riedel *et al.*, 1994).	Lactate utilisers (YELN) (propionibacteria)
Pal Propiobac-medium (Thierry & Madec, 1995).	Propionibacteria (Pal-P)
MEA-medium (Biolab) (pH 5.6) (Garrote *et al.*, 1997).	Yeasts (MEA)
YEC-medium (Biolab) (pH 6.0) (Rea *et al.*, 1996).	Yeasts (YEC)

[a] The following abbreviations were used: MRS = deMan, Rogosa and Sharpe-medium, KCA = Potassium Carboxymethyl Cellulose Agar, TTC = Triphenyltetrazolium Chloride, APM = Acetobacter Peroxydans Medium, YELN = Yeast Extract Lactate-medium with Naladixic acid, MEA = Malt Extract Agar, YEC = Yeast Extract Chloramphenicol-medium.

colonies should be selected from each plate for further identification. The Harrison Disc method (Harrigan & McCance, 1976) for the selection of microbial colonies permits more than one colony to be selected from an original plate in order to gain a fair representative of the microbes growing on a specific medium. Each colony selected from a specific medium was then numbered and streaked out on the different specific media in order to obtain pure cultures.

Microbial identification

Gram-staining, catalase and oxidase tests were performed on each of the colonies selected with the Harrison Disc method (Harrigan & McCance, 1976). After these initial tests, the colony characteristics were compared and those showing different colony and cell morphology or catalase, oxidase or Gram-stain characteristics, were further identified. The LAB were identified using the API 50 CHL (API system S.A., La Balme le Grottes, 38390, Montalieu Vercieu, France), while the yeasts were identified using the Rapid ID 32C (API system S.A., La Balme le Grottes, 38390, Montalieu Vercieu, France).

Microlog software (Biolog Inc., USA) was used to facilitate clustering and interpret the groupings of the microbes identified with the API 50 CHL and the Rapid ID 32 C identification systems. The data sets consisted of the characteristics of the isolated microbes as well as the characteristics of specific reference microbes (API system S.A., La Balme le Grottes, 38390, Montalieu Vercieu, France). One-dimensional plots were created on the basis of the calculated dendrogram differences (D_D) and the different isolates were clustered in relation to their closest relatives.

Results and discussion

The Kepi grains that were examined in this study can be divided into three different "groups". The first "group" consisted of Kepi grains that were locally produced and used to produce Kepi over a period of 30 d, using the normal method of Kepi production. Samples of these Kepi grains were then examined after 20, 25 and finally 30 d to obtain an indication of the microbes present in these specific grains. The second "group" of Kepi grains were also locally produced, but used to determine the impact of different stages in the production of Kepi and Kepi

grains on the microbial population of the grains. This study was done in triplicate. Firstly, all of the grains were activated for three days, after which samples of the grains were removed and examined. The rest of the Kepi grains were then subjected to 'mass production conditions' (Schoevers, 2000) where yeast extract and urea were added to the milk and the milk incubated for 10 d. Again samples of the Kepi grains were removed and examined. Finally, the remaining Kepi grains were used to produce Kepi, using the normal method of Kepi production, for 30 d, after which yet more samples of the grains were removed and examined. The third "group" of Kepi grains consisted of the eight samples of Kepi grains obtained from other sources (Table 1). Each sample of Kepi grains was activated for 24 h and each then examined.

For each sample of Kepi grains the following was determined: the enumeration values of the microbes present in the grains; the identification of the microbes present in the grains; the distribution frequency of the identified microbes; and the inter- and intra-relationships between these microbes.

Enumeration values

Normal plating procedures are known to give an underestimation of the true microbial community as not all the microbes are recovered (Swanson *et al.*, 1992). This means that only a restricted part of the total genetic information of a community is, in fact, revealed. Since all plating procedures are selective and exclude part of the microbial community, seven different enumeration media were used in order to cover a wider spectrum of metabolic activity.

Enumeration values of locally produced Kepi grains at days 20, 25 and 30 of normal Kepi production (first "group")

The enumeration values ($cfu.g^{-1}$) of the isolated microbes on the various selective media (not including the Pal-P-medium) obtained from locally produced Kepi grains after days 20, 25 and 30, are given in Table 3. At all three time intervals (20, 25 and 30 d) higher enumeration values were obtained on the media used to select for LAB (MRS, KCA+TTC, KCA+V) and for the AAB (APM) than from the media used to select for propionibacteria (YELN) and yeasts (MEA, YEC) as can be seen in Fig. 1. At day 20 the highest numbers were obtained on the

Table 3. The enumeration values ($cfu.g^{-1}$) from locally produced Kepi grains after 20, 25 and 30 d of normal Kepi production.

Medium[a]	Sample 1 at 20 d	Sample 2 at 25 d	Sample 3 at 30 d
MRS	1.2×10^7	1.7×10^7	1.6×10^7
KCA+TTC	1.2×10^7	3.5×10^6	3.1×10^6
KCA+V	1.6×10^6	1.1×10^6	1.2×10^6
APM	1.4×10^7	3.2×10^6	2.2×10^6
YELN	2.6×10^5	1.6×10^5	1.0×10^5
MEA	1.8×10^5	1.4×10^4	1.1×10^4
YEC	4.3×10^4	1.6×10^4	9.0×10^4

[a] The following abbreviations were used: MRS = deMan, Rogosa and Sharpe-medium, KCA = Potassium Carboxymethyl Cellulose Agar, TTC = Triphenyltetrazolium Chloride, V = Vancomycin, APM = Acetobacter Peroxydans Medium, YELN = Yeast Extract Lactate-medium with Naladixic acid, MEA = Malt Extract Agar, YEC = Yeast Extract Chloramphenicol-medium.

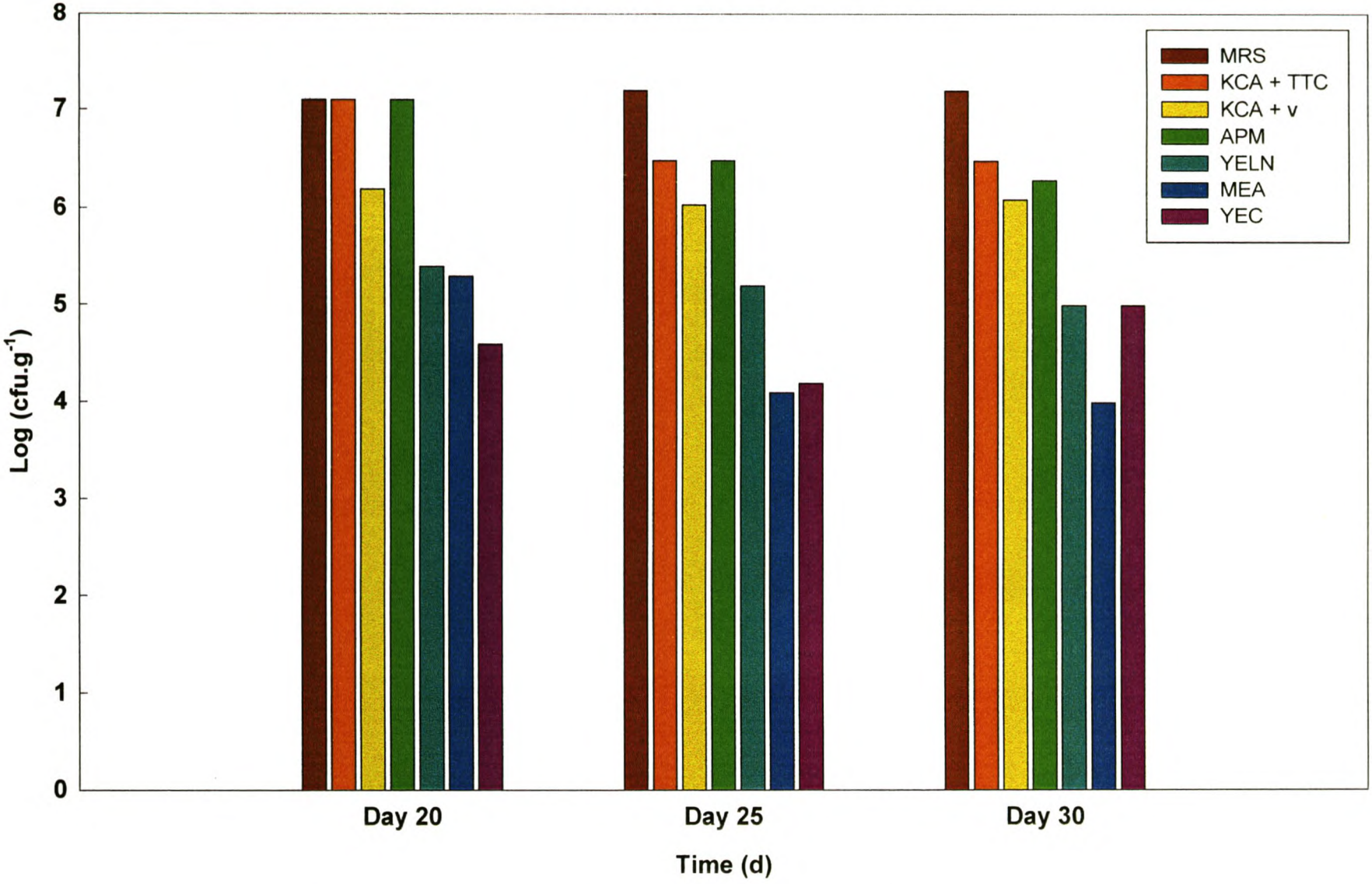

Figure 1. The microbial levels from locally produced Kepi grains at days 20, 25 and 30 on seven different selective media.

APM-medium (1.4 x 10^7 cfu.g^{-1}), followed by the MRS-medium and the KCA+TTC-medium (1.2 x 10^7 cfu.g^{-1}). At day 25, as well as on day 30, the highest numbers were obtained on the MRS-medium (1.7 x 10^7 and 1.6 x 10^7 cfu.g^{-1}, respectively). The numbers on the KCA+TTC-medium and on the APM-medium, at days 25 and 30, were somewhat lower. At day 25, 3.5 x 10^6 and 3.2 x 10^6 cfu.g^{-1} were obtained on the KCA+TTC-medium, and the APM-medium, respectively, while 3.1 x 10^6 and 2.2 x 10^6 cfu.g^{-1} were obtained from the same media at day 30. The values on the MEA and YEC-medium at days 20, 25 and 30 ranged from 1.1 x 10^4 to 1.8 x 10^5 cfu.g^{-1}, while the enumeration values on the YELN-medium for the three isolation times ranged from 1.0 x 10^5 to 2.6 x 10^5 cfu.g^{-1}. The enumeration values of these three media (MEA, YEC and YELN) are much lower than those obtained on the other media.

Enumeration values of locally produced Kepi grains after three days of activation, 10 d of mass production and a further 30 d of normal Kepi production (second "group")

This study was done in triplicate. The incubation time of the media for LAB (MRS, KCA+TTC and KCA+V) and propionibacteria (YELN and Pal-P) were lengthened from five to 10 d.

Control I - After three days of activation: The enumeration values (cfu.g^{-1}) from the Kepi grains, after three days of activation, are given in Table 4 and illustrated in Fig. 2A. The highest average enumeration value (2.2 x 10^8 cfu.g^{-1}) was obtained on the MRS-medium. The YELN-medium, APM-medium and KCA+TTC-medium had slightly lower average enumeration values at 1.9 x 10^8, 1.8 x 10^8 and 1.7 x 10^8 cfu.g^{-1}, respectively. Much lower average enumeration values were obtained on the KCA+V-medium (3.9 x 10^4 cfu.g^{-1}) and the Pal-P-medium (1.3 x 10^4 cfu.g^{-1}), while no growth was observed, in this study, on the YEC-medium.

Control II - After 10 d of mass production: The highest average enumeration value of the Kepi grains produced during the mass production procedure (1.6 x 10^8 cfu.g^{-1}) was obtained on the KCA+TTC-medium (Table 5). The average enumeration values obtained on the YELN-medium, MRS-medium and APM-medium were lower at 8.8 x 10^7, 5.9 x 10^7 and 1.8 x 10^7 cfu.g^{-1}, respectively. These enumeration values are somewhat lower (Fig. 2B) compared

Table 4. The enumeration values ($cfu.g^{-1}$) from Kepi grains after three days of Kepi grain activation.

Medium[a]	Sample 1	Sample 2	Sample 3
MRS	2.0×10^8	5.2×10^7	1.7×10^8
KCA+TTC	2.1×10^8	7.4×10^7	2.1×10^8
KCA+V	1.2×10^4	1.8×10^4	8.7×10^4
APM	2.6×10^8	1.3×10^8	1.5×10^8
YELN	2.1×10^8	1.7×10^8	1.8×10^8
Pal-P	4.6×10^3	2.8×10^4	6.0×10^3
YEC	0	0	0

Table 5. The enumeration values ($cfu.g^{-1}$) from Kepi grains after 10 d of mass production.

Medium[a]	Sample 1	Sample 2	Sample 3
MRS	1.8×10^7	1.4×10^8	1.9×10^7
KCA+TTC	1.6×10^8	1.9×10^8	1.3×10^8
KCA+V	1.3×10^6	1.6×10^7	2.0×10^7
APM	4.0×10^7	1.3×10^7	2.4×10^6
YELN	1.8×10^8	7.2×10^7	1.3×10^7
Pal-P	2.4×10^4	1.7×10^4	2.3×10^4
YEC	8.2×10^7	8.4×10^7	1.1×10^8

Table 6. The enumeration values ($cfu.g^{-1}$) from Kepi grains after 30 d of normal continuous Kepi production.

Medium[a]	Sample 1	Sample 2	Sample 3
MRS	7.6×10^6	8.1×10^6	1.1×10^7
KCA+TTC	3.1×10^7	2.7×10^7	3.2×10^7
KCA+V	8.0×10^6	1.1×10^7	1.0×10^7
APM	1.4×10^7	1.3×10^7	1.6×10^7
YELN	1.8×10^5	1.5×10^5	2.3×10^5
Pal-P	2.0×10^2	1.1×10^3	8.0×10^2
YEC	1.2×10^4	1.4×10^4	1.7×10^4

[a] The following abbreviations were used: MRS = deMan, Rogosa and Sharpe-medium, KCA = Potassium Carboxymethyl Cellulose Agar, TTC = Triphenyltetrazolium Chloride, V = Vancomycin, APM = Acetobacter Peroxydans Medium, YELN = Yeast Extract Lactate-medium with Naladixic acid, MEA = Malt Extract Agar, YEC = Yeast Extract Chloramphenicol-medium.

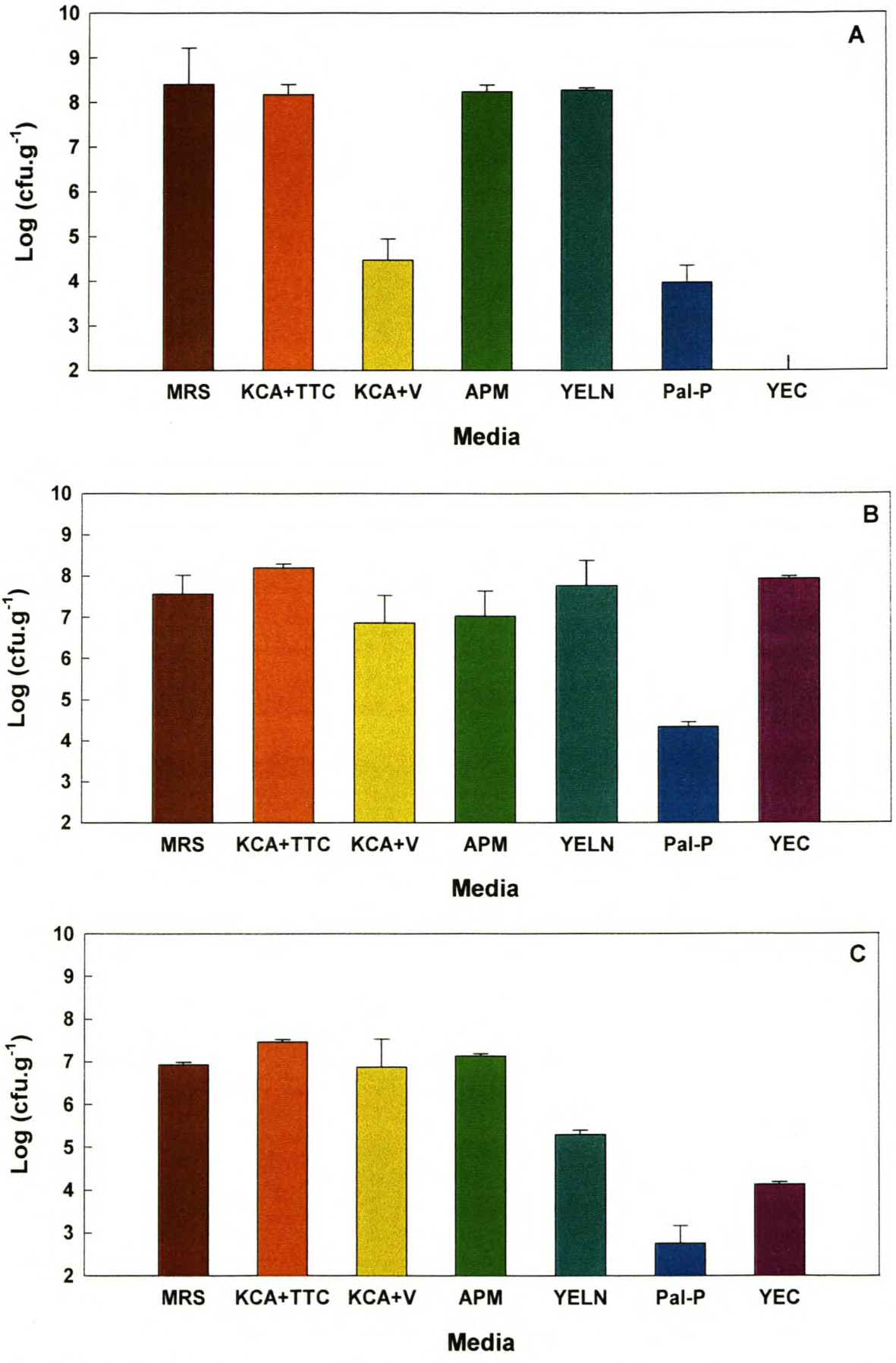

Figure 2. The microbial counts from locally produced Kepi grains after 3 d of activation (A),10 d of mass production (B) and 30 d of normal Kepi production (C) on seven different selective media. The standard deviation was used as the error-bar length

to those obtained on the same media from the Kepi grains that were activated for three days (Fig. 2A). The average enumeration values obtained on the YEC-medium (9.2 x 10^7 cfu.g^{-1}), KCA+V-medium (1.2 x 10^7 cfu.g^{-1}) and Pal-P-medium (2.1 x 10^4 cfu.g^{-1}), however, were higher than those obtained on the same media from the Kepi grains that were activated for three days. It is possible that the added yeast extract and urea and the environmental stress conditions applied during the mass production of the grains may have impacted the population structure leading to changes in the microbial numbers.

Control III - After a further 30 d of Kepi production: The data summarised in Table 6 and illustrated in Fig. 2C shows that the average enumeration values on all of the media decreased. According to the data, the highest average enumeration value (3.0 x 10^7 cfu.g^{-1}) was obtained on the KCA+TTC-medium as was also found in the case of the Kepi grains that were mass produced. Average enumeration values of 1.4 x 10^7, 1.0 x 10^7 and 8.9 x 10^6 cfu.g^{-1} were obtained on the APM-medium, KCA+V-medium and MRS-medium, respectively. The lowest average enumeration values were obtained on the YELN-medium (1.9 x 10^6 cfu.g^{-1}), YEC-medium (1.4 x 10^4 cfu.g^{-1}) and Pal-P-medium (7.0 x 10^2 cfu.g^{-1}).

This decrease in cfu.g^{-1} with time (three days of activation to 10 d of mass production to a further 30 d of Kepi production) possibly suggests that the longer Kepi grains are used to produce Kepi, the lower the microbial levels may be. It is possible that, at an early stage of Kepi production, the right combination of microbes are not yet formed for optimum polysaccharide (kefiran) production. Kefiran production had, therefore, not reached an optimum concentration and might not be holding the microbes very tightly together, making them easier to isolate. It is also possible that the concentration of microbes in the grains slowly decreased naturally the longer Kepi grains were produced on an uninterrupted basis.

Enumeration values from Kepi grains obtained from different sources (third "group")

The Kepi grains from various sources (Table 1) were activated in double pasteurised full cream milk for only 24 h in order to prevent the grains from changing their specific microbial population composition as a result of the use of a

different type of milk and other culturing conditions. The enumeration values obtained from the various grains on the selected media are given in Table 7.

The counts (cfu.g^{-1}) for grain type-1 ranged from 2.4 x 10^5 (KCA+V-medium) to 8.9 x 10^5 (MEA-medium), while no growth was observed on the MRS-medium, YELN-medium and YEC-medium. The counts for grain type-2 varied more with values ranging between 6.4 x 10^4 (KCA + TTC-medium) and 4.2 x 10^6 (MRS-medium). No growth was found on the KCA+V and the YEC-media. In contrast to the previous two grain types, grain type-3 produced growth on all of the various media. The counts for this grain type varied from 3.2 x 10^6 (MRS-medium) to 3.7 x 10^8 (MEA-medium). Grain type-4 and grain type-5 produced no growth on the YELN and KCA+V-media, respectively, while the cfu.g^{-1} obtained on the rest of the media from grain type-4 ranged from 1.5 x 10^5 (YEC-medium) to 3.6 x 10^7 (KCA + TTC-medium) and that of grain type-5 ranged from 3.4 x 10^6 (KCA + TTC-medium) to 1.2 x 10^8 (APM-medium). Less variation was found in the enumeration values of grain type-6. The counts for this grain type ranged from 1.9 x 10^7 (YEC-medium) to 5.5 x 10^7 (MEA-medium). Again no growth was observed on the YELN and KCA+V-media. The counts for grain type-7 ranged from 1.1 x 10^7 (APM-medium) to 8.5 x 10^8 (YELN-medium). No growth was observed on the YEC-medium, as was also the case with grain types-1 and -2. Grain type-8, similar to grain type-3, produced growth on all of the media, with the counts ranging from 1.7 x 10^5 (APM-medium) to 4.2 x 10^7 (KCA+V-medium).

The occurrence of no observed growth on certain media could possibly be due to various factors. It has been reported on many occasions that the source of Kepi grains, the cultivation method and storage conditions play an important role in determining the microbial composition (Garrote *et al.*, 1997; Pintado *et al.*, 1996; Rašić, 1986). The specific microbes present in the grains will then, of course, determine on which medium growth will occur. In this study it was also found that the numerical numbers per medium varied from grain type to grain type (Fig. 3). In addition, the counts obtained on the various media showed a low variation in the case of grain types-1 and -6, while for other grain types, the counts varied much more (grain types-2, -3, -4, -5, -7 and -8).

Table 7. Enumeration values (cfu.g^{-1}) from Kepi grains obtained from eight different sources as determined with seven different media[a].

Grain type	MRS	KCA+TTC	KCA+V	APM	YELN	MEA	YEC
1	0	5.0×10^5	2.4×10^5	7.3×10^5	0	8.9×10^5	0
2	4.2×10^6	6.4×10^4	0	9.9×10^5	2.7×10^6	4.3×10^5	0
3	3.2×10^6	1.6×10^7	3.6×10^6	1.7×10^8	1.7×10^7	3.7×10^8	1.9×10^7
4	1.3×10^7	3.6×10^7	6.6×10^6	1.2×10^6	0	4.0×10^6	1.5×10^5
5	7.9×10^6	3.4×10^6	0	1.2×10^8	8.1×10^7	6.3×10^7	1.9×10^7
6	4.2×10^7	4.9×10^7	0	4.7×10^7	0	5.5×10^7	1.9×10^7
7	1.4×10^7	6.5×10^7	5.3×10^7	1.1×10^7	8.5×10^8	5.5×10^7	0
8	1.1×10^6	7.2×10^6	4.2×10^7	4.0×10^5	1.8×10^6	1.7×10^5	6.1×10^5

[a] The following abbreviations were used: MRS = deMan, Rogosa and Sharpe-medium, KCA = Potassium Carboxymethyl Cellulose Agar, V = Vancomycin, APM = Acetobacter Peroxydans Medium, YELN = Yeast Extract Lactate-medium with Naladixic acid, MEA = Malt Extract Agar, YEC = Yeast Extract Chloramphenicol-medium.

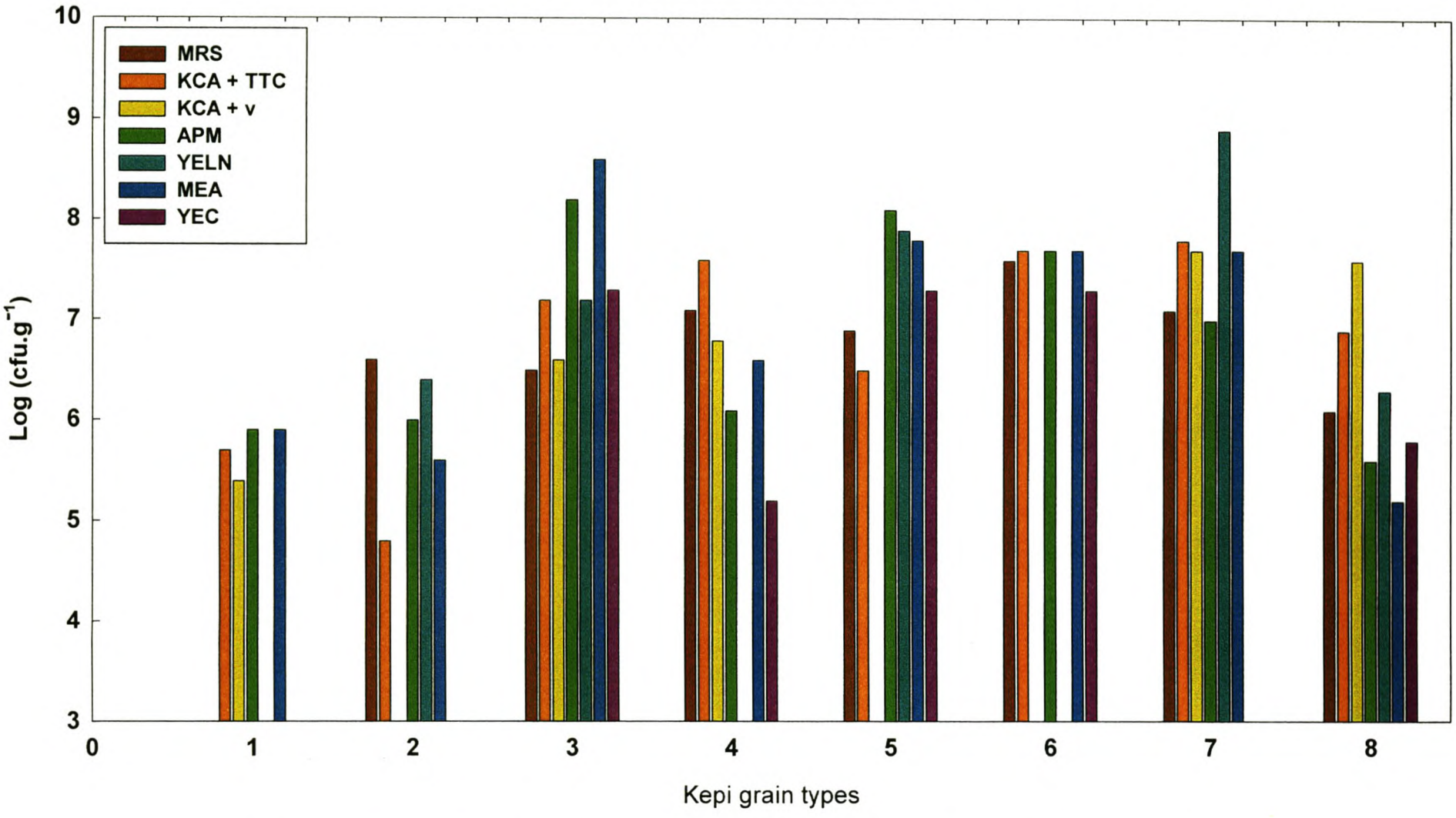

Figure 3. The microbial counts from Kepi grains obtained from other sources (Table 1) using seven selective media.

Identification of isolates

As a result of the large volume of data generated during the morphological and biochemical characterising of the isolates using the API 50 CHL and Rapid ID 32C systems together with the results of the additional tests performed, the data is given at the end of this chapter in Appendix A (Tables A1, A2, A3, A4, A5, A6, A7, A8 and A9). This was done to simplify the discussion section.

Identification of isolates from locally produced Kepi grains after 20, 25 and 30 days of normal Kepi production (first "group")

In this study, the microbial strains present in locally produced Kepi grains after 20, 25 and 30 d of normal Kepi production were determined and are given in Table 8. After 20 d of Kepi production the following LAB were identified: *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis 2* and *Lactobacillus brevis 3*. The yeasts included a strain of *Cryptococcus humicolus* and *Zygosaccharomyces*. After 25 d of Kepi production, the LAB identified were *Leuc. mesenteroides* subsp. *cremoris*, *Lc. lactis* subsp. *lactis 2* and *Lb. fermentum*. *Cryptococcus humicolus* was the only yeast species identified after 25 days. The LAB present after 30 d included *Leuc. mesenteroides* subsp. *cremoris* and *Lb. fermentum*, while *Cryptococcus humicolus* was again the only yeast species found.

The grains examined in this study did not reveal the presence of acetic acid bacteria (AAB), as reported by Rosi (1978) and Koroleva (1988). Pintado *et al.* (1996) also found no AAB in the Kepi grains they studied. These microbes are considered contaminants by some workers (Angulo *et al.*, 1993) as they are strict aerobes in what can essentially be seen as an anaerobic environment (Rea *et al.*, 1996).

Propionibacteria were also not found in any of the grains studied. The presence of propionibacteria has never been reported as part of the microbial population of Kepi grains or of the Kepi beverage. A possible reason for this is that, since they are bacteria that grow slowly (Perez-Chaia *et al.*, 1994b), they are not given sufficient time to develop once they are plated out. It might also be that the combination of microbes in certain grains do not allow for the propionibacteria to become a viable part of the microbial population of the grain. Furthermore, it is possible that they are present in such small numbers or bound by kefiran in

Table 8. Identification of the isolates present in Kepi grains after Kepi was produced over a period of 30 d.

Isolate number[a]	Identification	Isolation medium	Identification (%)	Identification acceptability
20 d				
L1 1MS	*Leuc. mesenteroides* subsp. *cremoris*	MRS	83.7	Acceptable
L1 1KV	*Leuc. mesenteroides* subsp. *cremoris*	KCA+V	83.7	Acceptable
L1 1KT	*Lc. lactis* subsp. *lactis 2*	KCA+TTC	77.5	Acceptable
L1 2KV	*Lb. brevis 3*	KCA+V	93.3	Very good
Y1 1MA	*Zygosaccharomyces* sp.	MEA	97.4	Very good
Y1 1YC	*Cryptococcus humicolus*	YEC	92.7	Very good
Y1 3MA	*Cryptococcus humicolus*	MEA	92.5	Very Good
Y1 2YC	*Cryptococcus humicolus*	YEC	86.5	Acceptable
25 d				
L2 2KT	*Leuc. mesenteroides* subsp. *cremoris*	KCA+TTC	83.7	Acceptable
L2 1KV	*Leuc. mesenteroides* subsp. *cremoris*	KCA+V	83.7	Acceptable
L2 2KV	*Leuc. mesenteroides* subsp. *cremoris*	KCA+V	83.7	Acceptable
L2 3MS	*Lb. fermentum*	MRS	45.9	Doubtful
L2 4KV	*Lc. lactis* subsp. *lactis 2*	KCA+V	48.9	Doubtful
Y2 2YC	*Cryptococcus humicolus*	YEC	97.3	Very good
Y2 1YL	*Cryptococcus humicolus*	YELN	92.7	Very good
30 d				
L3 2KV	*Leuc. mesenteroides* subsp. *cremoris*	KCA+V	83.7	Acceptable
L3 1KV	*Leuc. mesenteroides* subsp. *cremoris*	KCA+V	83.7	Acceptable
L3 3MS	*Lb. fermentum*	MRS	49.8	Doubtful
Y3 2YC	*Cryptococcus humicolus*	YEC	86.5	Acceptable
Y3 3YC	*Cryptococcus humicolus*	YEC	92.7	Very good
Y3 1MA	*Cryptococcus humicolus*	MEA	86.5	Acceptable

[a] **Isolate number:** First two digits – sample number; third digit – isolated colony number; and last two digits – isolation medium (MS = MRS-medium, KT = KCA+TTC-medium, KV = KCA+V-medium, YL = YELN-medium, YC = YEC-medium, and MA = MEA-medium).

such a manner that they are not easily isolated. As for the influence propionibacteria have on the rest of the microbial population of Kepi grains, Liu & Moon (1983) reported that the addition of *Propionibacterium shermanii* to kefir inhibited the growth of LAB and yeasts, as well as the formation of ethanol. Similarly, Pérez-Chaia *et al.* (1994a) reported that, although low concentrations (0.7 $g.l^{-1}$) of propionic acid had no significant influence on the growth rate of lactobacilli, higher concentrations (6.7 $g.l^{-1}$) reduced both their growth rate and biomass yield.

The selectivity of the media used in this study for the isolation of the LAB (MRS, KCA+TTC and KCA+V-media) was found not to be as specific as expected, since specific species were isolated from more than one selective medium (Table 9). *Leuconostoc mesenteroides* subsp. *cremoris* was present on the MRS, KCA+TTC and KCA+V-media, while *Lc. lactis* subsp. *lactis 2* was isolated from both the KCA+TTC and the KCA+V-media. *Cryptococcus humicolus* was present on both the media selecting for yeasts (MEA and YEC), as well as on the medium selecting for the propionibacteria (YELN). This emphasises the importance of taking media selectivity into consideration when conclusions are made on the identity of microbes solely on enumeration values, without any further identification.

It is also important to remember that results concerning the microbial population of Kepi grains vary extensively. Özer & Özer (1999) reported the mesophilic homofermentative lactic streptococci (*Lc. lactis* subsp. *lactis* and *cremoris)* to be the most active components of Kepi grains and the mesophilic heterofermentative streptococci (*Leuc. mesenteroides*) to be mainly responsible for the development of the aroma, the characteristic taste of Kepi and possible gas formation in association with the yeasts. Furthermore, they reported that the *Lb. brevis* and *Lb. casei* subsp. *rhamnosus* species to be common in Kepi starters. Koroleva (1988), however, reported that the lactobacilli most frequently found in the grains are *Lb. kefir*, the obligate homofermentative *Lb. dulbrueckii* subsp. *bulgaricus* and *Lb. helveticus* and the facultative heterofermentative *Lb. casei* subsp. *rhamnosus*. In the case of yeasts, Marshall *et al.* (1984) found that the two most commonly isolated Kepi yeasts are *Candida kefyr* and *Saccharomyces cerevisiae*, while Pintado *et al.* (1996) reported *Sacch. delbrueckii* to be the yeast most frequently isolated from Kepi grains.

Table 9. The microbial structure of the Kepi grains at different stages of Kepi and Kepi grain production, as depicted by the growth medium[a].

Species	Number of isolates[a]							
	MRS	KCA+ TTC	KCA+ V	APM	YELN	Pal-P	MEA	YEC
Normal Kepi production (after 20, 25 and 30 d)								
Leuc. mesenteroides subsp. *cremoris*	1	1	5	-	-	-	-	-
Lb. brevis 3	-	-	1	-	-	-	-	-
Lb. fermentum	2	-	-	-	-	-	-	-
Lc. lactis subsp. *lactis 2*	-	1	1	-	-	-	-	-
Zygosaccharomyces spp.	-	-	-	-	-	-	1	-
Cryptococcus humicolus	-	-	-	-	1	-	2	5
After 3 d of activation								
Lb. fermentum	1	-	-	-	-	-	-	-
Lb. brevis 3	-	1	4	-	-	1	-	-
Lc. lactis subsp. *lactis 1*	1	3	-	3	1	1	-	-
After 10 d of mass production								
Lb. plantarum	3	2	2	-	-	2	-	-
Lc. lactis subsp. *lactis 1*	-	-	-	-	1	1	-	2
C. lambica	-	-	-	1	-	-	-	-
C. krusei	-	-	3	2	1	1	-	2
After 30 d of normal Kepi production								
Leuc. mesenteroides subsp. *cremoris*	-	3	3	-	-	-	-	-
Lb. fermentum	1	4	-	-	-	-	-	-
Lb. delbrueckii subsp. *delbrueckii*	3	-	-	-	-	2	-	-
C. kefyr	-	-	-	3	-	-	-	6

[a] The following abbreviations were used: MRS = deMan, Rogosa and Sharpe-medium, KCA = Potassium Carboxymethyl Cellulose Agar, TTC = Triphenyltetrazolium Chloride, V = Vancomycin, APM = Acetobacter Peroxydans Medium, YELN = Yeast Extract Lactate-medium with Naladixic acid, Pal-P = Pal Propiobac-medium, MEA = Malt Extract Agar, YEC = Yeast Extract Chloramphenicol-medium.

These contradictory results can be ascribed to the many factors that play an important role in establishing the unique microbial population of Kepi grains including the origin of Kepi grains, how the grains are handled, the type of milk used and the method by which Kepi is produced (Oberman & Libudzisz, 1998). In this study it was also found that although initial growth of certain colonies was observed on the media used for microbial isolation, these colonies did not grow when they were streaked out to obtain pure cultures. It could be possible that these colonies, although present in the grains, are not viable outside the rather complex Kepi grain environment.

Identification of isolates from locally produced Kepi grains after three days of activation, 10 d of mass production and a further 30 d of normal Kepi production (second "group")

The fact that this study was done in triplicate resulted in three types of samples of Kepi grains to be examined at each time interval, namely after three days of activation, 10 d of mass production and 30 d of normal Kepi production. This was done to determine the variation per grain sample.

Control I - After three days of activation: All three samples of Kepi grains were found to contain *Lc. lactis* subsp. *lactis 1* and *Lb. brevis 3* strains, as depicted in Table 10. Both these strains were able to grow on more than one specific growth medium (Table 9) and that, once again, emphasises the importance of using further identification criteria. Furthermore, sample one also contained *Lb. fermentum*. A mycelial fungus, *Geotrichum candidum*, was also isolated from all three samples. It has been reported that *G. candidum* is usually found on the surface of the Kepi grains, but does not seem to affect the performance of the grains or influence the organoleptic properties of the produced Kepi (Roginsky, 1988).

No yeasts were isolated from any of the three Kepi grain samples at this time interval. Similarly, in studies done in the Russian Federation on commercial Kepi it was reported that a small number or no yeasts were present in the Kepi grains (Barnett *et al.*, 1993). This occurrence might be explained by the fact that the yeasts might not have been introduced to the Kepi grains at this early stage (activation) or that, if they were present, they might just have been present in too low concentrations or it is also possible they were not yet active. Furthermore, the

Table 10. Identification of the isolates present in Kepi grains after an activation period of three days.

Isolate number[a]	Identification	Isolation medium	Identification (%)	Identification acceptability
Sample 1				
L1 1MS	*Lb. fermentum*	MRS	86.2	Acceptable
L1 2MS	*Lc. lactis* subsp. *lactis 1*	MRS	89.2	Acceptable
L1 1KT	*Lc. lactis* subsp. *lactis 1*	KCA+TTC	89.2	Acceptable
L1 3YL	*Lc. lactis* subsp. *lactis 1*	YELN	89.2	Acceptable
L1 1P	*Lc. lactis* subsp. *lactis 1*	Pal-P	89.2	Acceptable
L1 2A	*Lc. lactis* subsp. *lactis 1*	APM	89.2	Acceptable
L1 2KT	*Lb. brevis 3*	KCA+TTC	98.4	Very Good
L1 3KV	*Lb. brevis 3*	KCA+V	82.6	Acceptable
MF	*G. candidum*			
Sample 2				
L2 2KT	*Lc. lactis* subsp. *lactis 1*	KCA+TTC	89.2	Acceptable
L2 2A	*Lc. lactis* subsp. *lactis 1*	APM	89.2	Acceptable
L2 3KV	*Lb. brevis 3*	KCA+V	98.4	Excellent
L2 2P	*Lb. brevis 3*	Pal-P	98.4	Excellent
MF	*G. candidum*			
Sample 3				
L3 2KT	*Lc. lactis* subsp. *lactis 1*	KCA+TTC	89.2	Acceptable
L3 3A	*Lc. lactis* subsp. *lactis 1*	APM	89.2	Acceptable
L3 2KV	*Lb. brevis 3*	KCA+V	77.8	Acceptable
L3 3KV	*Lb. brevis 3*	KCA+V	77.8	Acceptable
MF	*G. candidum*			

[a] **Isolate number:** First two digits – sample number; third digit – isolated colony number; and last two digits – isolation medium (MS = MRS-medium, KT = KCA+TTC-medium, KV = KCA+V-medium, A = APM-medium, YL = YELN-medium, P = Pal-P-medium, YC = YEC-medium, and MA = MEA-medium).

structural relationship of the microbes in the grains could also have played an important role in the isolation of the yeasts. Bottazzi & Bianchi (1980) found that the peripheral part of the grain was mainly populated by bacteria, while the centre was dominated by yeasts. It is, therefore, possible that there might only have been a low concentration of yeasts present in the Kepi grains after three days of activation, but that their central position in the grains could have made them difficult to isolate. There were no propionibacteria or AAB isolated from the grains after three days of activation.

Control II - After 10 d of mass production: The microbes isolated from the Kepi grains after 10 d of mass production are given in Table 11. The isolates of sample one included *Lb. plantarum* and *Lc. lactis* subsp. *lactis 1*, while the yeasts species included *C. lambica* and *C. krusei*. Samples two and three of the Kepi grains contained the same bacteria namely *Lb. plantarum*, *Lc. lactis* subsp. *lactis 1* and one yeast species, *C. krusei*. The mycelial fungus, *G. candidum*, was isolated from all three samples.

It is possible that the yeast extract and urea that were added and the stress conditions applied during the mass production of the grains led to the dominant growth of *Lb. plantarum*, as this species was not isolated from Kepi grains at any other time interval. Klostermaier *et al.* (1999) reported that among the different nitrogen sources for *Lb. plantarum* that were tested, yeast extract was most important for this species, as the growth rate increased by 16% after the addition of yeast extract. Furthermore, neither *C. lambica* or *C. krusei* has previously been isolated as part of the microbial population of Kepi grains or the Kepi beverage, although both are frequently isolated from other dairy products (Barnett *et al.*, 1983). The addition of yeast extract and urea to the milk and the agitation applied during the mass production process could also have enhanced the growth of these two yeast species. It is also possible that the addition of yeast extract and urea as part of the mass production of the Kepi grains caused the increased numbers obtained on the KCA+V, the YEC and the Pal-P-media at this time interval as illustrated in Fig. 2B, since these were the media from which the yeasts were isolated. After the normal method of Kepi production was resumed for 30 d, the numbers obtained on all the media were found to decrease (Fig. 2C), supporting the believe that the added yeast extract and urea during the mass production could be responsible for the increased yeast growth.

Table 11. Identification of the isolates present in Kepi grains after 10 d of mass production.

Isolate number[a]	Identification	Isolation medium	Identification (%)	Identification acceptability
Sample 1				
L1 1MS	*Lb. plantarum*	MRS	99.9	Excellent
L1 2KT	*Lb. plantarum*	MRS	99.9	Excellent
L1 1KV	*Lb. plantarum*	KCA+V	99.9	Excellent
L1 3P	*Lb. plantarum*	Pal-P	99.9	Excellent
L1 1YC	*Lc. lactis subsp. lactis 1*	YEC	89.2	Acceptable
Y1 1A	*C. lambica*	APM	96.3	Very Good
Y1 1YC	*C. krusei*	YEC	98.3	Excellent
MF	*G. candidum*			
Sample 2				
L2 3MS	*Lb. plantarum*	MRS	99.9	Excellent
L2 1YL	*Lc. lactis* subsp. *lactis 1*	YELN	81.0	Acceptable
L2 1YC	*Lc. lactis* subsp. *lactis 1*	YEC	81.0	Acceptable
Y2 1KV	*C. krusei*	KCA+V	98.3	Excellent
Y2 2KV	*C. krusei*	KCA+V	98.3	Excellent
Y2 1P	*C. krusei*	Pal-P	98.3	Excellent
Y2 3A	*C. krusei*	APM	98.3	Excellent
MF	*G. candidum*			
Sample 3				
L3 1MS	*Lb. plantarum*	MRS	99.9	Excellent
L3 1KT	*Lb. plantarum*	KCA+TTC	99.9	Excellent
L3 2KV	*Lb. plantarum*	KCA+V	99.9	Excellent
L3 3P	*Lb. plantarum*	Pal-P	99.9	Excellent
L3 2P	*Lc. lactis* subsp. *lactis 1*	Pal-P	82.6	Acceptable
L3 2YC	*Lc. lactis* subsp. *lactis 1*	YEC	81.0	Acceptable
Y3 1KV	*C. krusei*	KCA+V	98.3	Excellent
Y3 1A	*C. krusei*	APM	98.3	Excellent
Y3 3YL	*C. krusei*	YELN	98.3	Excellent
Y3 2YC	*C. krusei*	YEC	98.3	Excellent
MF	*G. candidum*			

[a] **Isolate number:** First two digits – sample number; third digit – isolated colony number; and last two digits – isolation medium (MS = MRS-medium, KT = KCA+TTC-medium, KV = KCA+V-medium, A = APM-medium, YL = YELN-medium, P = Pal-P-medium, YC = YEC-medium, and MA = MEA-medium).

The identification of propionibacteria in this study was based on the change of the purple coloured Pal-P-medium to yellow, as a result of the production of propionic acid (Thièrry & Madec, 1995). This was found to be the case for the colonies that grew on the Pal Propiobac-medium after the Kepi grains were mass produced for 10 d. Further examination of the colonies by means of gas chromatography and Polymerase Chain Reaction analysis (see chapter four), however, resulted in a negative identification for propionibacteria. It was thus concluded that no members of the genus *Propionibacterium* were present.

There is clearly a definite change in the microbial population of the Kepi grains after three days of activation and after 10 d of mass production, indicating the impact of the mass production conditions on the microbial population. It was also found that all the isolates from these grains, with the exception of *C. lambica*, were present on more than one specific medium, again indicating a lack of media selectivity.

Control III - After 30 d of normal Kepi production: The species composition identified from the triplicate samples was found to be the same (Table 12) and included *Lb. fermentum*, *Lb. delbrueckii* subsp. *delbrueckii* and *Leuc. mesenteroides* subsp. *cremoris*. A single yeast species, *C. kefyr*, was identified. The data also showed that all the isolates were again able to grow on more than one specific medium (Table 9).

In this study the grains did not contain the mycelial fungus *G. candidum*. It is possible that *G. candidum* is only found in the early stages of Kepi production, as it has been reported to only cover the grain surface (Roginsky, 1988). The colonies on the Pal Propiobac-medium were also found to decolour the purple medium to yellow. PCR and GC analysis were again carried out on the colonies isolated from the Pal Propiobac-medium and, again, negative results were obtained for propionibacteria. Further identification using the API 50 CHL system showed that these isolates were strains of *Lb. delbrueckii* subsp. *delbrueckii*. There were no members of the AAB present in the grains.

Identification of the isolates from Kepi grains from other sources (third "group")

The isolates from the grains from other sources (Table 1) are listed in Table 13. *Lactobacillus delbrueckii* subsp. *delbrueckii* and a *Zygosaccharomyces* strain were the only species present in grain type-1. A *Lactobacillus delbrueckii*

Table 12. Identification of the isolates present in Kepi grains after kepi was produced for 30 d.

Isolate number[a]	Identification	Isolation medium	Identification (%)	Identification acceptability
Sample 1				
L1 2KT	*Lb. fermentum*	KCA+TTC	86.2	Acceptable
L1 4KT	*Lb. fermentum*	KCA+TTC	86.2	Acceptable
L1 1KV	*Leuc. mesenteroides* subsp. *cremoris*	KCA+V	78.4	Acceptable
L1 2KT	*Leuc. mesenteroides* subsp. *cremoris*	KCA+TTC	78.4	Acceptable
L1 2P	*Lb. delbrueckii* subsp. *delbrueckii*	Pal-P	93.1	Very good
L1 3MS	*Lb. delbrueckii* subsp. *delbrueckii*	MRS	93.1	Very good
Y1 3A	*C. kefyr*	APM	99.7	Excellent
Y1 1YC	*C. kefyr*	YEC	99.7	Excellent
Y1 3YC	*C. kefyr*	YEC	99.9	Excellent
Sample 2				
L2 1MS	*Lb. fermentum*	MRS	45.9	Doubtful
L2 2KT	*Lb. fermentum*	KCA+TTC	86.2	Acceptable
L2 3KV	*Leuc. mesenteroides* subsp. *cremoris*	KCA+V	83.7	Acceptable
L2 3KT	*Leuc. mesenteroides* subsp. *cremoris*	KCA+TTC	83.7	Acceptable
L2 1P	*Lb. delbrueckii* subsp. *delbrueckii*	Pal-P	93.1	Very good
L2 4MS	*Lb. delbrueckii* subsp. *delbrueckii*	MRS	93.1	Very good
Y2 1A	*C. kefyr*	APM	99.7	Excellent
Y2 1YC	*C. kefyr*	YEC	99.7	Excellent
Y2 3YC	*C. kefyr*	YEC	99.9	Excellent
Sample 3				
L3 1KT	*Lb. fermentum*	KCA+TTC	45.9	Doubtful
L3 2KV	*Leuc. mesenteroides* subsp. *cremoris*	KCA+V	83.7	Acceptable
L3 4KT	*Leuc. mesenteroides* subsp. *cremoris*	KCA+TTC	83.7	Acceptable
L3 2MS	*Lb. delbrueckii* subsp. *delbrueckii*	MRS	93.1	Very good
Y3 3A	*C. kefyr*	APM	99.7	Excellent
Y3 2MA	*C. kefyr*	MEA	99.7	Excellent
Y3 1YC	*C. kefyr*	YEC	99.9	Excellent

[a] **Isolate number:** First two digits – sample number; third digit – isolated colony number; and last two digits – isolation medium (MS = MRS-medium, KT = KCA+TTC-medium, KV = KCA+V-medium, A = APM-medium, P = Pal-P-medium, YC = YEC-medium, and MA = MEA-medium).

Table 13. Identification of the isolates present in Kepi grains from other sources.

Isolate number[a]	Identification	Isolation medium	Identification (%)	Identification acceptability
Grain type-1				
L1 3KV	*Lb. delbrueckii* subsp. *delbrueckii*	YELN	99.1	Excellent
Y1 1A	*Zygosaccharomyces* sp.	APM	97.4	Very good
Grain type-2				
L2 1YL	*Lb. delbrueckii* subsp. *delbrueckii*	YELN	97.1	Very good
Grain type-3				
L3 2KT	*Leuc. lactis*	KCA+TTC	96.1	Very good
L3 3YL	*Lb. curvatus*	YELN	97.6	Very good
Y3 4A	*C. kefyr*	APM	99.9	Excellent
Y3 1MS	*C. kefyr*	MRS	99.9	Excellent
Y3 2MA	*C. lipolytica*	MEA	99.1	Excellent
Y3 2YC	*C. lipolytica*	YEC	99.1	Excellent
Grain type-4				
L4 3KT	*Lb. delbrueckii* subsp. *lactis*	KCA+TTC	91.0	Very good
L4 1KV	*Lb. delbrueckii* subsp. *lactis*	KCA+V	91.0	Very good
Y4 2MS	*C. kefyr*	MRS	99.7	Excellent
Y4 1KT	*C. kefyr*	KCA+TTC	99.7	Excellent
Grain type-5				
L5 2KT	*Lc. lactis* subsp. *lactis 1*	KCA+TTC	76.5	Acceptable
L5 3KT	*Lb. delbrueckii* subsp. *delbrueckii*	KCA+TTC	99.1	Excellent
L5 1YL	*Lb. delbrueckii* subsp. *delbrueckii*	YELN	99.1	Excellent
Y5 3YC	*C. lipolytica*	YEC	99.1	Excellent
Y5 4YC	*C. lipolytica*	YEC	99.1	Excellent
Grain type-6				
L6 2KT	*Lb. delbrueckii* subsp. *lactis*	KCA+TTC	91.0	Very good
L6 3KT	*Lb. delbrueckii* subsp. *lactis*	KCA+TTC	91.0	Very good
Y6 1MS	*C. kefyr*	MRS	99.7	Excellent
Y6 3YC	*Saccharomyces cerevisiae*	YEC	99.9	Excellent
Y6 4YC	*C. lipolytica*	YEC	99.6	Excellent

Table 13. (continued)

Isolate number[a]	Identification	Isolation medium	Identification (%)	Identification acceptability
Grain type-7				
L7 3MS	*Leuc. mesenteroides* subsp. *mesenteroides/ dextranicum 2*	MRS	99.9	Excellent
L7 1KV	*Leuc. mesenteroides* subsp. *mesenteroides/ dextranicum 2*	KCA+V	99.9	Excellent
L7 3KV	*Leuc. mesenteroides* subsp. *mesenteroides/ dextranicum 2*	KCA+V	99.9	Excellent
L7 1YL	*Leuc. mesenteroides* subsp. *mesenteroides/ dextranicum 2*	YELN	99.9	Excellent
Y7 3A	*C. holmii*	APM	95.4	Very good
Grain type-8				
L8 1MS	*Lb. fermentum*	MRS	99.1	Excellent
L8 3MS	*Lb. fermentum*	MRS	98.3	Very good
L8 3KT	*Lb. fermentum*	KCA+TTC	98.3	Very good
L8 2KV	*Lb. fermentum*	KCA+V	98.3	Very good
L8 2KT	*Leuc. lactis*	KCA+TTC	99.4	Excellent
L8 3KV	*Leuc. lactis*	KCA+V	99.4	Excellent
Y8 3A	*C. holmii*	APM	95.4	Very good
Y8 3MA	*C. holmii*	MEA	95.4	Very good
Y8 1YC	*Zygosaccharomyces* sp.	YEC	97.4	Very good

[a] **Isolate number:** First two digits – sample number; third digit – isolated colony number; and last two digits – isolation medium (MS = MRS-medium, KT = KCA+TTC-medium, KV = KCA+V-medium, A = APM-medium, YL = YELN-medium, YC = YEC-medium, and MA = MEA-medium).

strain were the only species present in grain type-1. A *Lactobacillus delbrueckii* subsp. *delbrueckii* strain was the only species isolated from grain type-2. Grain type-3 resulted in a variety of microbes including *Lb. curvatus*, *Leuc. lactis*, *C. kefyr*, *C. valida* and *C. lipolytica*. *Lactobacillus delbrueckii* subsp. *delbrueckii* and *C. kefyr* were the only microbes isolated from grain type-4. *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lc. lactis* subsp. *lactis 1* and *C. lipolytica* were isolated from grain type-5. The isolates from grain type-6 included *Lb. delbrueckii* subsp. *lactis*, *Sacch. cerevisiae*, *C. kefyr* and *C. lipolytica*. Grain type-7 resulted in only two species, namely *Leuc. mesenteroides* subsp. *mesenteroides/dextranicum 2* and *C. holmii*. Grain type-8 resulted in a variety of isolates identified as *Lb. fermentum*, *Lb. brevis 3*, *Leuconostoc lactis*, *C. holmii* and a *Zygosaccharomyces* strain.

It is possible that the 24 h activation of the grains in a different type of milk than what had previously been used during Kepi production with these specific grains could have influenced the microbial structure of the grains and thus true Kepi was not made with these grains. This was done in order to prevent prolonged subjection to the local type of milk. It is also possible that the age of these various grains could also have influenced the microbial populations, but it was not possible to ascertain the age of these eight grain types.

Lactobacilli were present in each grain type with the exception of grain type-7, which again indicates the importance of this group in the production of Kepi. It was also frequently found that even though microbial growth was initially observed in the extracts of some grain types, these colonies were not viable outside the Kepi grain environment and, therefore, did not grow on the selected media. No AAB or propionibacteria were found in any of these Kepi grains from other sources. It is interesting to note that a strain of *Lb. curvatus*, isolated from grain type-3, has never previously been reported as a member of any Kepi grains.

Distribution frequencies

The distribution frequency of the prevalent LAB and yeast strains from the locally produced Kepi grains at the three time intervals, namely 20, 25 and 30 days of Kepi production, is shown in Fig. 4. At each time interval the total percentage of LAB was more than the total percentage of yeast.

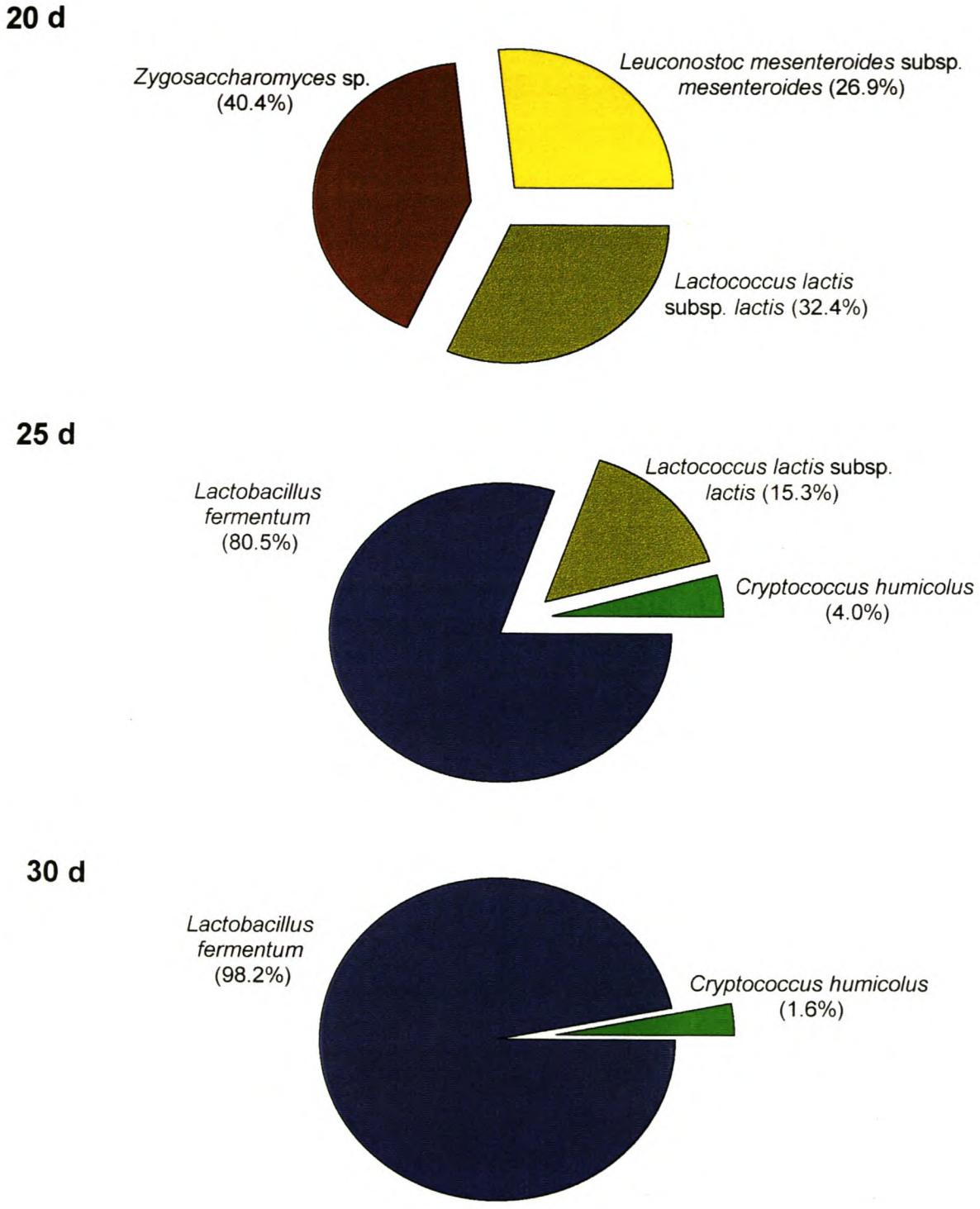

Figure 4. The distribution frequency of the prevalent microbial species in Kepi grains on days 20, 25 and 30 of normal Kepi production. Strains that were present in the Kepi grains at a concentration of less than 1.0% were excluded.

The impact of factors such as the duration and method of Kepi production on the microbial population of the grains can be seen in the average distribution frequencies of the prevalent LAB and yeast strains from locally produced Kepi grains after three days of activation, 10 d of mass production and 30 d of normal Kepi production (Fig. 5A - 5C). The data showed that LAB were again the prevalent microbes at all times, and this was taken as an indication of the integral role this group plays during the production of Kepi. In Portuguese Kepi grains, as studied by Pintado *et al.* (1996), LAB were also found to dominate. More specifically, Angulo *et al.* (1993) reported that, of the LAB group, the genus *Lactobacillus* is the most frequently found microbe in Kepi grains.

The distribution frequency of the prevalent isolates from Kepi grains obtained from other sources, after a 24 h activation period, is shown in Fig. 6. In contrast to the previous data, the yeast percentage obtained from some grain types was more than the percentage LAB (grain types-1, -3, -4 and -7). This is especially contradictory to the data regarding the microbial composition obtained from locally produced Kepi grains after activation where no yeasts were isolated (Table 10). This could possibly reflect the impact the source of the grains has on the microbial population.

However, it must be remembered that the microbes illustrated in these figures are not the only microbes that were present in the Kepi grains at each time interval, since all plating procedures are selective and exclude part of a specific microbial community. The microbial species depicted in the figures represents the dominant (numerically dominant) species that were isolated from the Kepi grains each time. Furthermore, the method does not allow any microbes present in the Kepi grains at a low concentration (<1.0%) to be included in the figures.

Inter- and intra-relationships

The morphological and biochemical characteristics of the isolates, as well as the data of the additional tests, used to facilitate the clustering of related bacteria and the subsequent plotting of one-dimensional dendrograms, are summarised in Appendix A (Tables A1, A2, A3, A4, A5, A6, A7, A8 and A9). The relationship positions of the major groups including known reference strains (Table

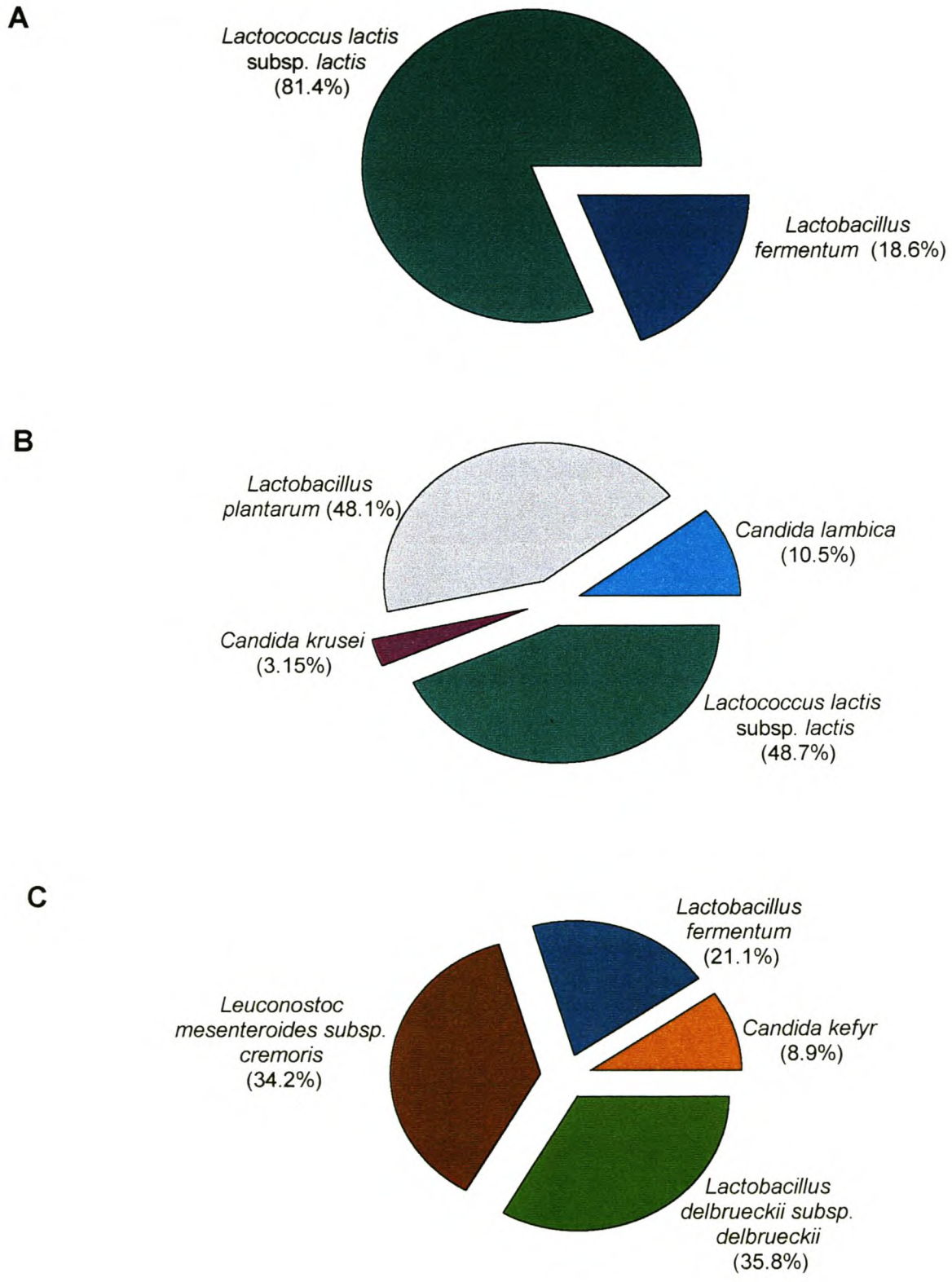

Figure 5. The average distribution frequency of the prevalent microbial population of Kepi grains after three days of activation (A), 10 d of mass production (B) and 30 d of normal Kepi production (C). Strains present in the Kepi grains at a concentration of less than 1.0% were excluded.

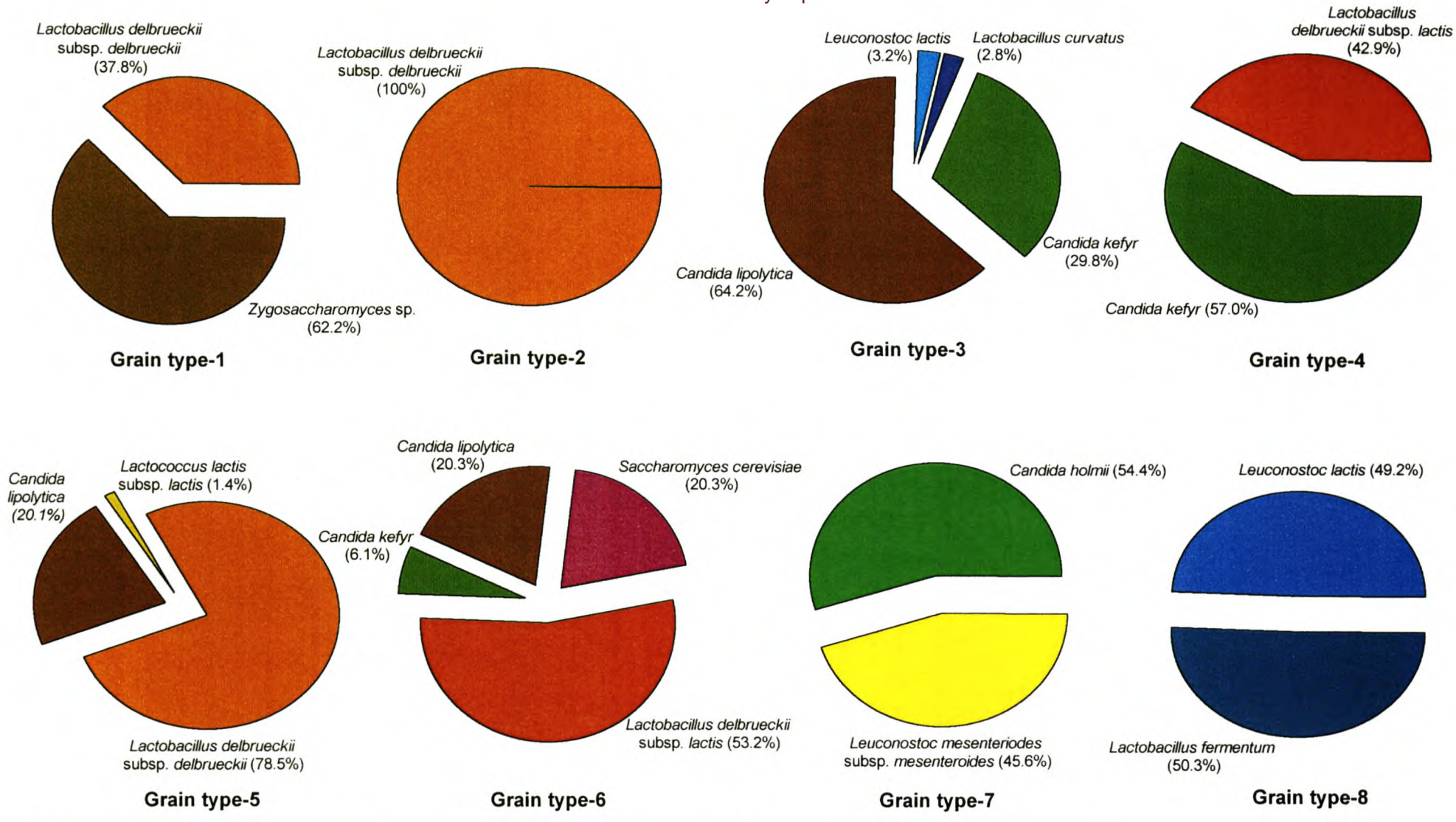

Figure 6. The distribution frequency of the prevalent microbial population from Kepi grains obtained from other sources (Table 1). Strains that were present in the Kepi grains at a concentration of less than 1.0% were excluded.

14), are illustrated as dendrograms in Fig. 7 and 8. The clustering groupings obtained are as follows:

Lactic Acid Bacteria (Fig. 7): Nine major groups, with several minor groups, were found for the LAB isolates and reference strains (Table 14) during the dendrogram analysis. The first group (group-1) consisted of a *Lb. delbrueckii* subsp. *delbrueckii* strain (L21YL), the *Lb. delbrueckii* subsp. *lactis* strains with the reference strain L7, the *Leuc. lactis* strains and the *Leuc. mesenteroides* subsp. *cremoris* reference strain L10, indicating a fairly close relationship between these two groups of strains. The *Lb. delbrueckii* subsp. *lactis* strains were clustered at a distance of three D_D from the reference strain L7, showing a high similarity. The reference strain of the *Lb. delbrueckii* subsp. *delbrueckii* isolate (L6) and *Leuc. lactis* strains (L11) were placed in different groups (5 and 6), respectively. However, the *Lb. delbrueckii* subsp. *delbrueckii* strain had a "very good" API identification of 97.1%, while the two *Leuc. lactis* strains had "very good" to "excellent" API identifications at 96.1 and 99.4%, respectively. A possible explanation for this phenomena where the reference strains are placed elsewhere is the occurrence of "wild" strains in nature which do not have many similar metabolic characteristics as that of the reference strains. It must also be taken into consideration that the reference strains are only a guide to which unknown strains are compared so as to confirm the identification status.

Group-2 contained three *Lb. brevis 3* strains clustered at a distance of either two or six D_D from the reference strain L3, as well as the *Lb. fermentum* strains and the reference strain for *Lb. fermentum* (L9). The *Lb. fermentum* strains were clustered at a distance of either six or seven D_D from the reference strain L9 which would explain the "doubtful" (49.6 and 43.1%) and only "acceptable" (86.2%) API results for eight of the strains. However, four of the *Lb. fermentum* strains had API identifications of either 98.3 or 99.1%, but were still placed at the same D_D distances from the reference strain as the strains with lower API identifications. As mentioned, these are the kind of problems that might arise with "wild" strains when they are compared to "reference laboratory" strains and which, subsequently, make the final identifications very difficult.

Group-3 contained a single *Lb. brevis 3* strain (L12KV) which "cluster lapped" to group-2 and all of the *Leuc. mesenteroides* subsp. *cremoris* strains, although the reference strains for both *Lb. brevis 3* (L3) and *Leuc. mesenteroides*

Table 14. Reference strains used during the dendrogram clustering.

Dendrogram number	Strain
Lactic acid bacteria	
L1	*Lb. brevis 1*
L2	*Lb. brevis 2*
L3	*Lb. brevis 3*
L4	*Lb. plantarum 1*
L5	*Lb. plantarum 2*
L6	*Lb. delbrueckii* subsp. *delbrueckii*
L7	*Lb. delbrueckii* subsp. *lactis*
L8	*Lb. curvatus*
L9	*Lb. fermentum*
L10	*Leuc. mesenteroides* subsp. *cremoris*
L11	*Leuc. lactis*
L12	*Leuc. mesenteroides* subsp *mesenteroides/dextranicum 2*
L13	*Leuc. mesenteroides* subsp *mesenteroides/dextranicum 1*
L14	*Lc. lactis* subsp. *lactis 1*
L15	*Lc. lactis* subsp. *lactis 2*
Yeasts	
Y1	*Zygosaccharomyces* sp.
Y2	*Cryptococcus humicolus*
Y3	*C. lambica*
Y4	*C. krusei*
Y5	*C. kefyr*
Y6	*C. lipolytica*
Y7	*Sacch. cerevisiae*
Y8	*C. holmii*

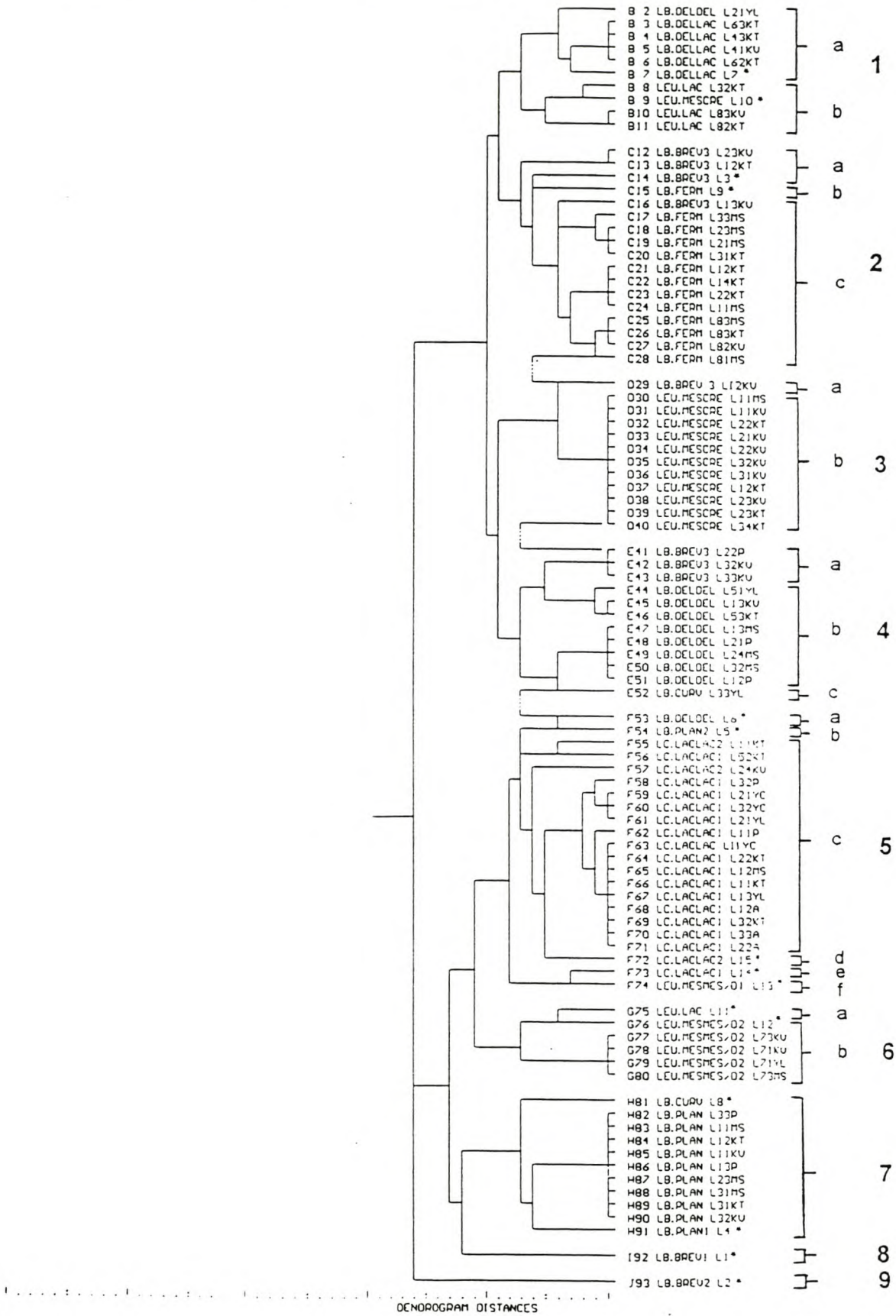

Fig. 7. Dendrogram indicating the clustering of the LAB strains in relation to the reference strains*

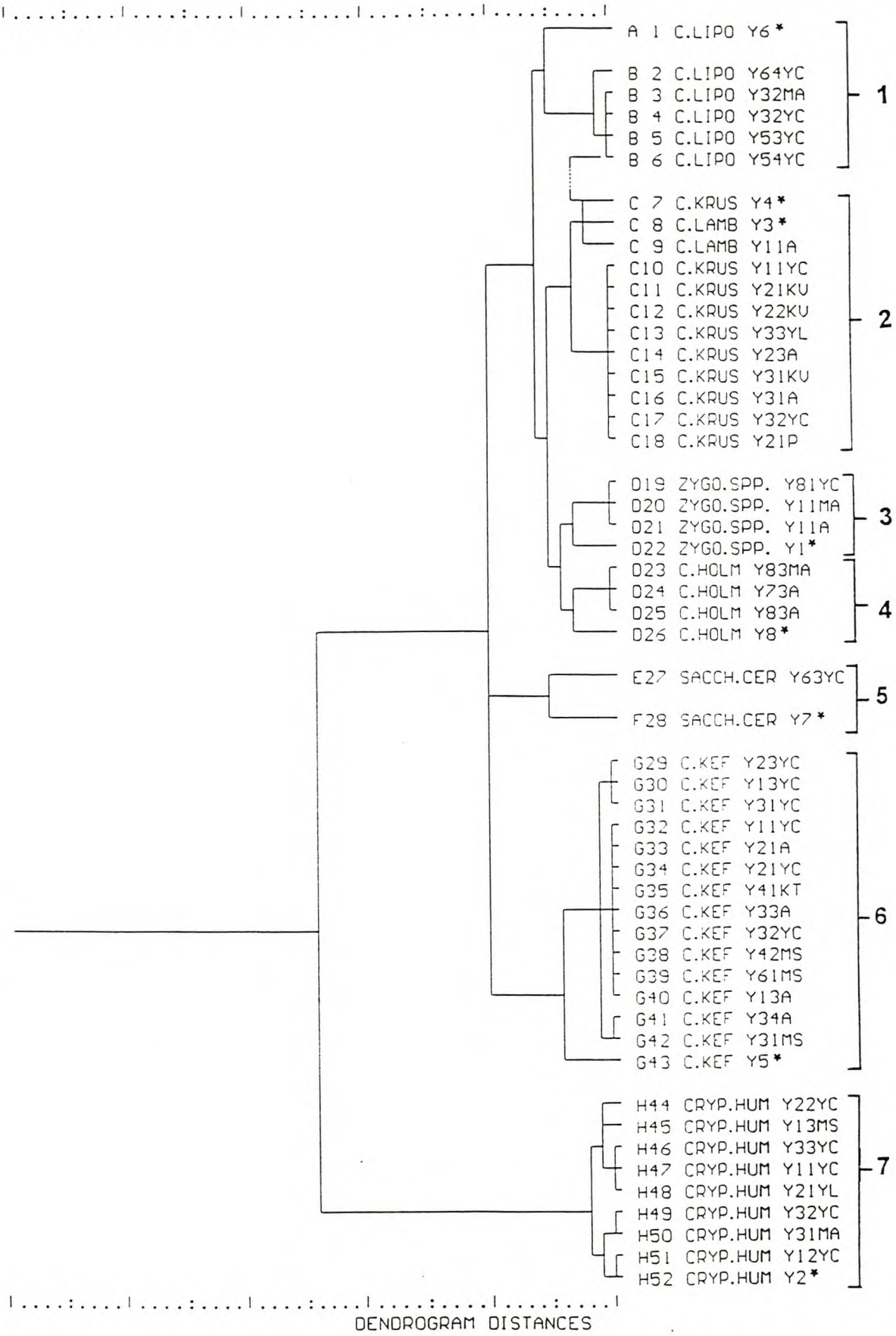

Fig. 8. Dendrogram indicating the clustering of the yeast strains in relation to the reference strains*.

subsp. *cremoris* (L10) were placed in different groups (groups-2 and -3, respectively). The *Lb. brevis 3* strain in this group (group-3) nevertheless had a "very good" API identification of 93.3%. The API identifications for the *Leuc. mesenteroides* subsp. *cremoris* strains were found to be "acceptable" at 78.4%, although the strains were also placed far from the reference strain, L10 (group-1).

Group-4 consisted of three more *Lb. brevis 3* strains, the *Lb. delbrueckii subsp. delbrueckii* strains and a single *Lb. curvatus* strain (L33YL). The *Lb. brevis 3* strains were clustered far from the reference strain L3, in group-2, which supports their only "acceptable" API identification of 77.8%. The *Lb. delbrueckii* subsp. *delbrueckii* strains were found to be "lap clustered" with the reference strain L6 (group-5), explaining the "very good" to "excellent" API identifications of either 93.1 or 99.1%. The *Lb. curvatus* strain in this group (group-4) showed a similar clustering arrangement with a "very good" API identification of 97.6%, but clustered far from its reference strain, L8 (group-7).

Group-5 contained five reference strains namely L5, L6, L13, L14 and L15, together with the strains of *Lc. lactis* subsp. *lactis 1* and *Lc. lactis* subsp. *lactis 2*. The *Lc. lactis* subsp. *lactis 1* strains were in the same main cluster with the reference strain L14, and "acceptable" API identification results of 76.5 - 89.2% was found. The two *Lc. lactis* subsp. *lactis 2* strains (L11KT and L24KV) were placed further from the reference strain L15. The API 50 CHL data bank, however, only gave an "acceptable" (77.5%) and even "doubtful" (48.9%) identification result for these two strains respectively. This is possibly due to main differences in the characteristics of these strains compared to that of the reference strain.

Group-6 contained the reference strain for *Leuc. lactis*, L11, as well as the *mesenteroides* subsp. *mesenteroides/dextranicum 2* strains and the reference strain L12. The *Leuc. mesenteroides* subsp. *mesenteroides/dextranicum 2* strains were thus fairly near their reference strain, having "excellent" API identifications of 99.9%.

Besides the reference strain for *Lb. curvatus* (L8), group-7 also contained the *Lb. plantarum* strains with the reference strain L4. However, the *Lb. plantarum* strains showed a fair D_D distance from their reference strain L4, despite having an "excellent" API identification of 99.9%. It is important to remember that the reference strains are only a guide to the identity of unknown strains in order to

confirm the identification status. Group-8 and group-9 each contained only a single reference strain, L1 and L2, respectively, with no *Lb. brevis 1* or *Lb. brevis 2* strains being isolated from any Kepi grains.

Yeasts (Fig. 8): The yeast isolates and reference strains (Table 14) were clustered into seven major groups. Group-1 consisted of a single reference strain for *C. lipolytica* (Y6), with the *C. lipolytica* strains placed together next to the reference strain but with several differences in D_D. The *C. lipolytica* strains all had "excellent" identification results of >99.0% with the API Rapid ID 32 C system, although they were not placed directly next to their reference strain (Y6). This again illustrates the differences between "wild" environmental isolates and reference strains.

Group-2 consisted of the *C. krusei* strains with the reference strain Y4, as well as the *C. lambica* strain with its reference strain Y3, indicating a close relationship between these strains. The *C. krusei* strains were clustered at a fairly close distance of three D_D from the reference strain Y4, which is in agreement with the "very good" API identification result of 98.3% for these strains. The single *C. lambica* isolate was placed at the same D_D distance as its reference strain Y3, indicating high similarity. This also correlates with the high API identification of 96.3% for the strain.

Group-3 contained the *Zygosaccharomyces* strains, as well as the reference strain for *Zygosaccharomyces* (Y1). The *Zygosaccharomyces* strains, with an API identification of 97.4%, were placed at a fairly close distance of three D_D from the reference strain Y1, while in group-4 the *C. holmii* strains, with an API identification of 95.4%, were also placed at a distance of three D_D from their reference strain (Y8).

The only strain of *Sacch. cerevisiae* isolated in this study was placed in group-5, with the reference strain for *Sacch. cerevisiae* (Y7). However, the *Sacch. cerevisiae* strain had an "excellent" API identification of 99.9%, but showed some variation to its reference strain (Y7).

Group-6 consisted of all the *C. kefyr* strains and the reference strain Y5. The *C. kefyr* strains were clustered at a distance of four D_D from the reference strain, with API identifications being >99.0%.

Group-7 contained the *Cryptococcus humicolus* strains which clustered closely to the reference strain Y2. However, for this group the API identifications

for the *Cryptococcus humicolus* strains varied from "acceptable" (86.5%) to "very good" (92.5, 92.7 and 97.3%).

In general though, there was much less strain variation between the yeast groups and their respective reference strains. Pintado *et al.* (1996) reported that, in the Portuguese Kepi grains they studied, the yeasts were the least affected by environmental conditions. This apparent stability of yeast strains obtained from the "environment" can possibly be an indication of the stability of their biochemical characteristics.

Conclusions

The results obtained in this study clearly demonstrated that the microbial content of Kepi grains varies as the methods used during Kepi and Kepi grain production changes (Table 15). The counts were found to decrease the longer Kepi was produced on an uninterrupted basis, except for when yeast extract and urea were added to the milk during the mass production of the grains. No yeasts were isolated from Kepi grains after three days of activation, but the addition of yeast extract and urea resulted in a clear increase in yeast numbers. The yeast isolates were identified as *C. lambica* and *C. krusei* and together with *Lactobacillus plantarum*, they were not isolated from Kepi grains produced at any other stage of Kepi production. Furthermore, Kepi grains obtained from different sources were also composed of different microbes (Table 13), confirming that the origin and history of Kepi grains probably strongly influenced the microbial composition.

The data also clearly indicated that it is very important to take the degree of medium selectivity into account when conclusions are made about the identity of the isolated microbes solely on whether microbes grow or do not grow on a specific medium, without any further identification. In this study, certain microbes were able to grow on more than just one specific medium (Table 9), which only became apparent when the isolates were further identified using the API 50 CHL and the Rapid ID 32C identification systems. This further identification of the isolates prevented any misinterpretation of the composition of the microbial population of Kepi grains.

In this study, the selective medium, Pal Propiobac, was used to identify propionibacteria in Kepi grains. However, after the isolates from the selective

Table 15. The microbial composition of locally produced Kepi grains at various stages of Kepi and Kepi grain production.

Control I – After three days of Kepi grain activation	**Control II – After 10 d of mass production**	**Control III – After 30 d of normal Kepi production**
Lb. fermentum	-	*Lb. fermentum*
Lb. brevis 3	-	-
Lc. lactis subsp. *lactis 1*	*Lc. lactis* subsp. *lactis 1*	-
-	-	*Lb. delbrueckii* subsp. *delbrueckii*
-	-	*Leuc. mesenteroides* subsp. *cremoris*
-	-	*C. kefyr*
-	*Lb. plantarum*	-
-	*C. lambica*	-
-	*C. krusei*	-

medium were tested by means of gas chromatography and Polymerase Chain Reaction methods, a negative result for propionibacteria were found. Further identification using the API 50 CHL system showed the specific isolates to be *Lb. delbrueckii* subsp. *delbrueckii*. This emphasises the importance of confirming the identity of isolated microbes when selective media are used for identification purposes.

The data obtained in this study, therefore, clearly indicates that the microbial composition of Kepi grains can not be defined without taking into account factors such as the method of Kepi production, the origin of the grains and the method of microbial identification. These factors all contribute to the variation that is found in the microbial population of Kepi grains.

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APPENDIX A

To Chapter Three

Tables A1 - A9 are given in this appendix. This large amount of data was placed in a separate appendix to simplify the discussion section of this chapter.

Table A1. Characterisation (API 50 CHL) of the LAB isolates from Kepi grains after Kepi was continuously produced over a period of 30 d.

Test	L1 1MS	L1 1KV	L1 1KT	L1 2KV	L2 2KT	L2 1KV	L2 2KV	L2 3MS	L2 4KV	L3 2KV	L3 1KV	L3 3MS
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+
Morphology	Cocci	Cocci	Cocci	Rod	Cocci	Cocci	Cocci	Rod	Cocci	Cocci	Cocci	Rod
Catalase	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation of:												
Control	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	+	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	+	+	-	-	-	+	-	-	-	+
Ribose	-	-	+	-	-	-	-	?	+	-	-	+
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	?
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-
ß Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	?	?	+	+	?	?	?	+	+	?	?	+
D-Glucose	-	-	+	-	-	-	-	+	-	-	-	+
D-Fructose	-	-	-	-	-	-	-	+	-	-	-	-
D-Mannose	-	-	+	-	-	-	-	-	-	-	-	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D-mannoside	-	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D-glucoside	-	-	-	-	-	-	-	-	-	-	-	-
N Acetyl glucosamine	-	-	+	-	-	-	-	-	+	-	-	-
Amygdaline	-	-	-	-	-	-	-	-	+	-	-	-
Arbutine	-	-	+	-	-	-	-	-	+	-	-	-
Esculine	+	+	+	+	+	+	+	+	+	+	+	+
Salicine	-	-	+	-	-	-	-	-	+	-	-	-
Cellobiose	-	-	+	-	-	-	-	-	+	-	-	-
Maltose	-	-	+	-	-	-	-	+	+	-	-	+
Lactose	-	-	+	-	-	-	-	+	+	-	-	+
Melibiose	-	-	-	-	-	-	-	+	+	-	-	+
Saccharose	-	-	-	-	-	-	-	+	-	-	-	+
Trehalose	-	-	+	-	-	-	-	-	+	-	-	-
Inuline	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-	-	-	-
Amidon	-	-	-	-	-	-	-	-	-	-	-	-

Table A1 (continued).

Test	L1 1MS	L1 1KV	L1 1KT	L1 2KV	L2 2KT	L2 1KV	L2 2KV	L2 3MS	L2 4KV	L3 2KV	L3 1KV	L3 3MS
Glycogene	-	-	+	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-
ß Gentiobiose	-	-	+	-	-	-	-	-	+	-	-	-
D-Turanose	-	-	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	+	+	-	-	-	+	-	-	-	+
2 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-
5 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-

Table A2. Characterisation (Rapid ID 32C) of the yeast isolates from Kepi grains after Kepi was continuously produced over a period of 30 d.

Test	Y1 1MA	Y1 1YC	Y1 3MS	Y1 2YC	Y2 2YC	Y2 1YL	Y3 2YC	Y3 3YC	Y3 1MA
Gram stain	+	+	+	+	+	+	+	+	+
Morphology	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-
Fermentation of:									
Galactose	-	+	+	+	+	+	+	+	+
Actidione	-	+	+	+	+	+	+	+	+
Saccharose	-	+	+	+	+	+	+	+	+
N Acetyl glucosamine	-	+	+	+	+	+	+	+	+
DL-lactate	-	+	+	+	+	+	+	+	+
L-Arabinose	-	+	+	+	+	+	+	+	+
Cellobiose	-	+	+	+	+	+	+	+	+
Raffinose	-	+	+	+	+	+	+	+	+
Maltose	-	+	+	+	+	+	+	+	+
Trehalose	-	+	+	+	+	+	+	+	+
2 ceto-gluconate	-	+	+	+	+	+	+	+	+
Methyl-D-glucoside	-	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+
D-Xylose	-	+	+	+	+	+	+	+	+
Ribose	-	+	+	+	+	+	+	+	+
Glycerol	-	+	+	+	+	+	+	+	+
Rhamnose	-	+	+	+	+	+	+	+	+
Palatinose	-	+	+	+	+	+	+	+	+
Erythritol	-	+	+	+	+	+	+	+	+
Melibiose	-	+	+	+	+	+	+	+	+
Glucuronate	-	+	+	+	+	+	+	+	+
Melezitose	-	+	+	+	+	+	+	+	+
Gentiobiose	-	+	+	+	+	+	+	+	+
Lévulinate	-	+	+	+	+	+	+	+	+
Mannitol	-	+	+	+	+	+	+	+	+
Lactose	-	+	-	+	+	+	+	+	+
Inositol	-	-	-	+	-	-	+	-	+
Glucose	+	+	+	+	+	+	+	+	+
Sorbose	-	+	+	+	-	+	+	+	+
Glucosamine	-	+	+	+	+	+	+	+	+
Esculine	-	-	-	+	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-

Table A3. Characterisation (API 50 CHL) of the LAB isolates from Kepi grains after an activation period of three days.

Test	L1 1MS	L1 2MS	L1 1KT	L1 3YL	L1 2A	L1 2KT	L1 3KV	L2 2KT	L2 2A	L2 3KV	L2 2P	L3 2KT	L3 3A	L3 2KV	L3 3KV
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Morphology	Rod	Cocci	Cocci	Cocci	Cocci	Rod	Rod	Cocci	Cocci	Rod	Rod	Cocci	Cocci	Rod	Rod
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation of:															
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+	-	-	-	-	+	+	-	-	+	+	-	-	-	-
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	+	+	+	+	-	-	+	+	-	-	+	+	+	+
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ß Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
D-Glucose	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-
D-Fructose	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-
D-Mannose	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D-mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N Acetyl glucosamine	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-
Amygdaline	-	+	+	+	+	-	-	+	+	-	-	+	+	-	-
Arbutine	-	+	+	+	+	-	-	+	+	-	-	+	+	-	-
Esculine	-	+	+	+	+	-	-	+	+	-	-	+	+	+	+
Salicine	-	+	+	+	+	-	-	+	+	-	-	+	+	-	-
Cellobiose	-	+	+	+	+	-	-	+	+	-	-	+	+	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	-	+	+	+	-	-	+	+	-	-
Melibiose	+	-	-	-	-	+	-	-	-	+	+	-	-	-	-
Saccharose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	-	+	+	+	+	-	-	+	+	-	-	+	+	+	+
Inuline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amidon	-	+	+	+	+	-	-	+	+	-	-	+	+	-	-
Glycogene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A3 (continued).

Test	L1 1MS	L1 2MS	L1 1KT	L1 3YL	L1 2A	L1 2KT	L1 3KV	L2 2KT	L2 2A	L2 3KV	L2 2P	L3 2KT	L3 3A	L3 2KV	L3 3KV
ßGentiobiose	-	+	+	+	+	-	-	+	+	-	-	+	+	-	-
D-Turanose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	+	-	-	-	-	+	+	-	-	+	+	-	-	-	-
2 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A4. Characterisation (API 50 CHL) of the LAB isolates from Kepi grains after 10 d of mass production.

Test	L1 1MS	L1 2KT	L1 1KV	L1 3P	L1 1P	L1 1YC	L2 3MS	L2 1YL	L2 1YC	L3 1MS	L3 1KT	L3 2KV	L3 3P	L3 2P	L3 2YC
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Morphology	Rod	Rod	Rod	Rod	Cocci	Cocci	Rod	Cocci	Cocci	Rod	Rod	Rod	Rod	Cocci	Cocci
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation of:															
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	+	+	-	+	+	-	-	-	-	+	+
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ß Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-
Sorbitol	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-
α Methyl-D-mannoside	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-
α Methyl-D glucoside	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-
N Acetyl glucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdaline	+	+	+	+	-	+	+	-	-	+	+	+	+	-	-
Arbutine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculine	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+
Salicine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	+	+	-	+	+	-	-	-	-	+	+
Melibiose	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Saccharose	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inuline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amidon	-	-	-	-	+	+	-	+	+	-	-	-	-	+	+
Glycogene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A4. (continued).

Test	L1 1MS	L1 2MS	L1 1KV	L1 3P	L1 1P	L1 1YC	L2 3MS	L2 1YL	L2 1YC	L3 1MS	L3 1KT	L3 2KV	L3 3P	L3 2P	L3 2YC
ßGentiobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Turanose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 ceto-gluconate	-	-	-	-	-	-	-	-	-	-		-	-	-	-
5 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A5. Characterisation (Rapid ID 32C) of the yeast isolates from Kepi grains after 10 d of mass production.

Test	Y1 1A	Y1 1YC	Y2 1Kv	Y2 2Kv	Y2 1P	Y2 3A	Y3 1Kv	Y3 1A	Y3 3YL	Y3 2YC
Gram stain	+	+	+	+	+	+	+	+	+	+
Morphology	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-
Fermentation of:										
Galactose	-	-	-	-	-	-	-	-	-	-
Actidione	-	-	-	-	-	-	-	-	-	-
Saccharose	-	-	-	-	-	-	-	-	-	-
N Acetyl glucosamine	+	+	+	+	+	+	+	+	+	+
DL-lactate	+	+	+	+	+	+	+	+	+	+
L-Arabinose	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-
2 ceto-gluconate	-	-	-	-	-	-	-	-	-	-
Methyl-2-glucoside	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-
D-Xylose	+	-	-	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-
Palatinose	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-
Glucuronate	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	-	-	-
Lévulinate	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+
Sorbose	-	+	+	+	+	+	+	+	+	+
Glucosamine	-	-	-	-	-	-	-	-	-	-
Esculine	-	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-

Table A6. Characterisation (API 50 CHL) of the LAB isolates from Kepi grains after Kepi was continuously produced over a period of 30 d.

Test	L1 2KT	L1 4KT	L1 1KV	L1 2KT	L1 2P	L1 3MS	L2 1MS	L2 2KT	L2 3KV	L2 3KT	L2 1P	L2 4MS	L3 1KT	L3 2KV	L3 4KT	L3 2MS
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Morphology	Rod	Rod	Cocci	Cocci	Rod	Rod	Rod	Rod	Cocci	Cocci	Rod	Rod	Rod	Cocci	Cocci	Rod
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation of:																
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+	+	-	-	-	-	+	+	-	-	-	-	+	-	-	-
Ribose	+	+	-	-	+	+	?	+	-	-	+	+	?	-	-	+
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ß Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	?	?	-	-	+	+	?	?	-	-	+	?	?	-
D-Glucose	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	+
D-Fructose	-	-	-	-	+	+	+	-	-	-	+	+	+	-	-	+
D-Mannose	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D-mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N Acetyl glucosamine	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	+
Amygdaline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arbutine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculine	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Salicine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	+
Maltose	+	+	-	-	-	-	+	+	-	-	-	-	+	-	-	-
Lactose	+	+	-	-	-	-	+	+	-	-	-	-	+	-	-	-
Melibiose	+	+	-	-	-	-	+	+	-	-	-	-	+	-	-	-
Saccharose	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inuline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amidon	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycogene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A6. (continued).

Test	L1 2KT	L1 4KT	L1 1KV	L1 2KT	L1 2P	L1 3MS	L2 1MS	L2 2KT	L2 3KV	L2 3KT	L2 1P	L2 4MS	L3 1KT	L3 2KV	L3 4KT	L3 2MS
ßGentiobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Turanose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
2 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A7. Characterisation (Rapid ID 32C) of the yeast isolates from Kepi grains after normal continuous production of Kepi over a period of 30 d.

Test	Y1 3A	Y1 1YC	Y1 3YC	Y2 1YC	Y2 1MA	Y2 3YC	Y3 3A	Y3 2YC	Y3 1YC
Gram stain	+	+	+	+	+	+	+	+	+
Morphology	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-
Fermentation of:									
Galactose	+	+	+	+	+	+	+	+	+
Actidione	+	+	+	+	+	+	+	+	+
Saccharose	+	+	+	+	+	+	+	+	+
N Acetyl glucosamine	-	-	-	-	-	-	-	-	-
DL-lactate	+	+	+	+	+	+	+	+	+
L-Arabinose	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-
Raffinose	+	+	+	+	+	+	+	+	+
Maltose	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-
2 ceto-gluconate	-	-	-	-	-	-	-	-	-
Methyl-2-glucoside	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-
Palatinose	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-
Glucuronate	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-
Gentiobiose	-	-	-	-	-	-	-	-	-
Lévulinate	-	-	-	-	-	-	-	-	-
Mannitol	+	+	-	+	+	-	+	+	-
Lactose	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+
Sorbose	-	-	-	-	-	-	-	-	-
Glucosamine	-	-	-	-	-	-	-	-	-
Esculine	+	+	+	+	+	+	+	+	+
Control	-	-	-	-	-	-	-	-	-

Table A8. Characterisation (API 50 CHL) of the LAB isolates from Kepi grains from various sources.

Test	L1 3KV	L2 1YL	L3 2KT	L3 3YL	L4 3KT	L4 1KV	L5 2KT	L5 3KT	L5 1YL	L6 2KT	L6 3KT	L7 3MS	L7 1KV	L7 3KV	L7 1YL	L8 1MS	L8 3MS	L8 2KV	L8 3KT	L8 2KT	L8 3KV
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Morphology	Rod	Rod	Cocci	Rod	Rod	Rod	Cocci	Rod	Rod	Rod	Rod	Cocci	Cocci	Cocci	Cocci	Rod	Rod	Rod	Rod	Cocci	Cocci
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation of:																					
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-
Ribose	+	+	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ß Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
D-Mannose	-	+	-	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D-mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D glucoside	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
N Acetyl glucosamine	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+
Amygdaline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arbutine	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculine	+	-	-	+	-	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-
Salicine	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Saccharose	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+
Trehalose	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Inuline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Amidon	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Glycogene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A8. (continued).

Test	L1 3KV	L2 1YL	L3 2KT	L3 3YL	L4 3KT	L4 1KV	L5 2KT	L5 3KT	L5 1YL	L6 2KT	L6 3KT	L7 3MS	L7 1KV	L7 3KV	L7 1YL	L8 1MS	L8 3MS	L8 2Kv	L8 3KT	L8 2KT	L8 3KV
ßGentiobiose	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Turanose	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-
2 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A9. Characterisation (Rapid ID 32C) of the yeast isolates from Kepi grains from different sources.

Test	Y1 1A	Y3 4A	Y3 1MS	Y3 2MA	Y3 2YC	Y4 2MS	Y4 1KT	Y5 3YC	Y5 4YC	Y6 1MS	Y6 3YC	Y6 4YC	Y7 3A	Y8 3A	Y8 3MA	Y8 1YC
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Morphology	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation of:																
Galactose	-	+	+	-	-	+	+	-	-	+	-	-	+	+	+	-
Actidione	-	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-
Saccharose	-	+	+	-	-	+	+	-	-	+	+	-	+	+	+	-
N Acetyl glucosamine	-	-	-	+	+	-	-	+	+	-	-	+	-	-	-	-
DL-lactate	-	+	+	-	-	+	+	-	-	+	+	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	+	+	-	-	+	+	-	-	+	+	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
2 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-2-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	-	-	+	+	-	-	+	-	-	-	-	-	+
D-Xylose	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Glycerol	-	-	-	+	+	-	-	+	+	-	-	+	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Palatinose	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Erythritol	-	-	-	+	+	-	-	+	+	-	-	+	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucuronate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mélézitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lévulinate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	+	+	-	-	+	+	-	-	+	-	-	-	-	-	-
Lactose	-	+	+	-	-	+	+	-	-	+	+	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucosamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculine	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

CHAPTER 4

IMPACT OF DIFFERENT PACKAGING CONDITIONS ON THE MICROBIAL COMMUNITY OF KEPI GRAINS

Abstract

Kepi is a cultured milk product that is produced by a mixed acidic and alcoholic fermentation. This fermentation is attributed to the microbes that are found within the Kepi grains that are used to produce Kepi. In this study, the impact of different packaging conditions on the microbial community of laboratory produced Kepi grains, was determined. Kepi grains were lyophilised, packaged in three different packaging materials ("low-density polyethylene film" (LDPE) "oriented polyester film" (OPET) and "methallised oriented polyester film" (MOPET)) and stored for two months at room temperature. Selective media, morphology and physiological characteristics were used to obtain the enumeration values and identify the microbes that constituted the grains that were subjected to lyophilisation and packaging. The Kepi grains packaged in OPET resulted in slightly higher microbial counts than that of the grains packaged in LDPE and MOPET. The starter organisms isolated from the grains included *Lactobacillus delbrueckii* subsp. *lactis* (LDPE, OPET and MOPET), *Lb. brevis* (OPET), *Lb. curvatus* (MOPET).

Introduction

Kepi is a self-carbonated, fermented milk beverage that has its origin in the Caucasus Mountains in the former Soviet Union (Duitschaever, 1989; Kurmann *et al.*, 1992; Saloff-Coste, 1999). This slightly acidic beverage enjoys a rich tradition of health claims (Gates, 1996; Saloff-Coste, 1999), as it is generally considered to have a high nutritional, biological and dietetic value (Libudzisz *et al.*, 1990). In addition to this, one of the functions of the microbes constituting the Kepi grains includes the production of lactic acid and other biological active components (Kaufmann, 1997) that increase the storage capability of the milk and inhibits the growth of undesirable and possible pathogenic microbes (Libudzisz *et al.*, 1990; Steinkraus, 1996; Takahashi & Kawakami, 2001; Van Wyk, 2001).

Traditionally Kepi production involved the fermentation of milk in leather bags or oak barrels (Libudzisz *et al.*, 1990). This led to the build up of layers of microbes, which were usually embedded in protein and polysaccharide, and could be recovered as a solid grainy matrix (Roginsky, 1988). The microbes in Kepi grains exist in a complex symbiotic relationship and primarily include lactic acid bacteria (LAB) and yeasts (Fujisawa *et al.*, 1988; Libudzisz *et al.*, 1990; Özer & Özer, 1999; Saloff-Coste, 1999; Steinkraus, 1996). Rosi (1978) also described the presence of acetic acid bacteria (AAB), but ascribed their presence to a lack of asepsis during the Kepi manufacturing process. It is known that many environmental factors influence the microbial content of the Kepi grains, such as the type of milk used during the fermentation, the origin and storage of the grains and the method by which Kepi is produced (Garrote *et al.*, 1997; Pintado *et al.*, 1996; Rašić, 1986).

Another factor that influence the Kepi grain microbial community is the method that is used to store and preserve the grains. Methods include storage at room temperature, refrigeration, freezing and lyophilisation (Vedamuthu, 1982). Garrote *et al.* (1997) reported that storage at -80°C altered the microbial composition of Kepi grains less than when storage was done at either -20°C or -4°C. However, milk fermented with grains that had been stored at -80°C produced a Kepi beverage similar to the milk fermented with control grains, whereas grains stored at -4°C produced a beverage with unpleasant characteristics (Garrote *et al.*, 1997) suggesting a major change in the microbial community.

A better understanding of the various microbes constituting the Kepi grains and how they can be stored without major changes to the microbial community will undoubtedly lead to a more efficient grain preservation method. The aim of this study was to determine the impact of different environmental conditions including lyophilisation and storage in a "low density polyethylene film" (LDPE), an "oriented polyester film" (OPET) and a "methallised oriented polyester film" (MOPET) at room temperature for two months, on the microbial community of laboratory produced Kepi grains.

Materials and methods

Starter cultures

The Kepi grains used in this study were obtained from the Department of Food Science, University of Stellenbosch, South Africa.

Activation of Kepi grains and Kepi production

Eighteen grams of Kepi grains were added to 500 ml of double pasteurised full cream milk for activation, followed by incubation at 25°C. The grains were separated by sieving and placed in fresh milk every 24 h until a good quality beverage was produced and then the microbial community of the grains was studied.

Mass production

The mass production of the Kepi grains was done according to the method developed by Schoevers (2000) and patented (SA Patent 2000/1896). The procedure involved the addition of 2% (w/v) yeast extract (Biolab) and 0.5% (w/v) urea (Biolab) to 400 ml of double pasteurised full cream milk. Forty grams of activated Kepi grains were then added to the pasteurised milk mixture and the containers incubated at 25°C in a shake waterbath. The grains were sieved out and placed in a new milk mixture containing the additional yeast extract and urea every 24 h.

Packaging studies

The lyophilisation and packaging of the Kepi grains as described in this study, were done in collaboration with A. Cilliers (personal communication, 2001). Laboratory produced Kepi grains were divided into 18 g units and lyophilised in a Hetto CT freeze drier (Denmark) for 4 d. The Kepi grain units (which had a mass of 4 g each after lyophilisation) were then packaged and sealed using three different packaging materials and stored in desicators at room temperature for two months. The packaging materials included a "low density polyethylene film" (LDPE), an "oriented polyester film" (OPET) and a "methallised oriented polyester film" (MOPET) (Mr. F. Hannay, 2000, Nampak R&D, Cape Town, personal communication). The moisture vapour transmission rates (MVTR) and oxygen

permeability values per day, as well as the costs (R per kg) of each of the packaging films, are given in Table 1.

Isolation and identification of the microbial community

The microbes present in the Kepi grains were isolated by homogenizing 10 g of Kepi grains in 90 ml of sterile saline solution (SSS) (0.85% (w/v) NaCl) using a Stomacher (BagMixer, Interscience, France) for 15 min. Serial dilutions in SSS of 10^{-1} to 10^{-6} were plated on the different selective media (as described in Chapter 3 of this thesis) given in Table 2. The media used for the selection of the LAB (MRS, KCA+TTC and KCA+V) and the propionibacteria (Pal-P) were incubated anaerobically using the Anaerocult A system (Merck) for 5 to 10 d at 30°C. Colonies selected from these plates for further identification were cultivated on MRS-medium. The media used for the selection of the yeasts (YEC) and the acetic acid bacteria (APM) were aerobically incubated for 3 to 5 d at 25°C. Any colonies selected from the media used for the selection of yeasts (YEC) were further cultivated on PDA, while colonies from the medium used to select for acetic acid bacteria (APM) were further cultivated on APM.

The Harrison Disc Method (Harrigan, 1998) was used for the random statistical selection of representative colonies. This method (Harrigan, 1998) permits more than one colony to be selected from an original plate in order to gain a fair representative of the microbes growing on a specific medium. The selected colonies were purified and characterised using the Gram-stain, catalase and oxidase tests. These initial tests were used as basis for separating the morphologically different colonies, which were then further identified. The LAB were identified using the API 50 CHL system, while the yeasts were identified using the Rapid ID 32C system (API system S.A., La Balme le Grottes, 38390 Montalieu Vercieu, France). Although API systems do not contain data banks that are comprehensive enough for the identification of all environmental or dairy isolates, it can still be used as a source for the determination of the physiological patterns of isolates.

Table 1. The moisture vapour transmission rates (MVTR) per day, oxygen permeability values per day and costs (R per kg) of "low density polyethylene film" (LDPE), "oriented polyester film" (OPET) and "methallised oriented polyester film" (MOPET).

Film	MVTR[a] ($g.m^{-2}$)	O_2 permeability[b] ($cm^3.m^{-2}$)	Costs (R per kg)
LDPE	5	2 000	15.06
OPET	9	110	38.64
MOPET	0.8	1.0	48.90

[a] Moisture vapour transmission rate measured at standard conditions of 38°C at 90% relative humidity.

[b] O_2 permeability measured at 23°C, 50% relative humidity and a pressure of 1 atm.

Table 2. Selective media used for the isolation and characterisation of the microbial strains present in Kepi grains.

Isolation medium [a]	**Selected microbes**
MRS-medium (Biolab) with 3% (w/v) ethanol (Merck) and 0.5% (w/v) filter sterilised cycloheximide (Merck) (pH 6.0) (Pintado *et al.*, 1996).	Lactobacilli (MRS)
KCA-medium ($g.l^{-1}$): tryptone (Biolab) 20.0; yeast extract (Saarchem) 5.0; gelatine (Merck) 2.5; glucose (Merck) 5.0; lactose (Merck) 5.0; sodium chloride (Saarchem) 4.0; tri sodium citrate.$2H_2O$ (Saarchem) 2.0; calcium lactate.$5H_2O$ (Saarchem) 8.0; agar (Biolab) 15.0; calcium citrate (Saarchem) 10.0 and carboxymethyl cellulose (Merck) (1.5% w/v) 100 ml. Ten milliltre filter sterilised TTC (Oxoid) was added (pH 6.6) (Nickels & Leesment, 1964).	Lactococci (KCA+TTC)
KCA-medium with 30 µg filter sterilized vancomycin (Fluka) (Benkerroum *et al.*, 1993) (pH 6.6).	Leuconostocs (KCA+V)
APM-medium ($g.l^{-1}$): malt extract (Biolab) 15.0; yeast extract (Saarchem) 5.0 and agar (Biolab) 15.0. Sixty millilitre filter sterilised ethanol (Merck) (50% v/v) was added (pH 6.8) (DSMZ, 2001).	Acetic acid bacteria (APM)
YELN-medium ($g.l^{-1}$): yeast extract (Biolab) 5.0; sodium lactate (Saarchem) (60% v/v) 20.0; agar (Biolab) 15.0 and Tween 80 (Merck) 1.0 ml. Ten millilitre filter sterilised naladixic acid (0.02%) was added (pH 7.2) (Riedel *et al.*, 1994)	Lactate utilisers (YELN) (propionibacteria)
Pal Propiobac-medium (Thièrry & Madec, 1995).	Propionibacteria (Pal-P)
MEA-medium (Biolab) (Garrote *et al.*, 1997) (pH 5.6).	Yeasts (MEA)
YEC-medium (Biolab) (Rea *et al.*, 1996) (pH 6.0).	Yeasts (YEC)
PDA-medium (Biolab) (Lin *et al.*, 1999) (pH 5.6).	Yeasts (PDA)

[a] The following abbreviations were used: MRS = deMan, Rogosa and Sharpe-medium, KCA = Potassium Carboxymethyl Cellulose Agar, TTC = Triphenyltetrazolium Chloride, APM = Acetobacter Peroxydans Medium, YELN = Yeast Extract Lactate-medium with Naladixic acid, MEA = Malt Extract Agar, YEC = Yeast Extract Chloramphenical-medium, PDA = Potato Dextrose Agar.

Results and Discussion

Control I - Microbial content of laboratory produced Kepi grains

The enumeration values ($cfu.g^{-1}$) obtained from normal Kepi grains after three days of activation are given in Table 3, and the species isolated are given in Table 4. The data used here as a normal Kepi population "Control" were as obtained and described in Chapter 3 of this thesis.

Control II - Microbial content of Kepi grains after 10 d of mass production

The enumeration values ($cfu.g^{-1}$) obtained from the Kepi grains after 10 d of mass production are given in Table 5, and the species isolated given in Table 6. The data used here as a Kepi grain mass production "Control" were as obtained and described in Chapter 3 of this thesis.

Microbial content of Kepi grains that were lyophilised and packaged in LDPE, OPET and MOPET

Enumeration values

The enumeration values from the Kepi grains packaged in the three different packaging materials and stored at room temperature for two months, are given in Table 7 and the data illustrated in Fig. 1A - C. After an activation period of three days, the highest average numbers obtained from the Kepi grains packaged in the "low density polyethylene film" (LDPE) were on the APM (2.3×10^6 $cfu.g^{-1}$) and KCA+V-media (1.7×10^6 $cfu.g^{-1}$). The average microbial counts obtained on the MRS-medium were lower at 7.3×10^5 $cfu.g^{-1}$, while those obtained on the KCA+TTC-medium were the lowest at 1.5×10^4 $cfu.g^{-1}$. No growth was found on the YELN, Pal-P and YEC-media.

The highest average counts obtained from the Kepi grains packaged in the "oriented polyester film" (OPET) were on the KCA+V (6.5×10^6 $cfu.g^{-1}$) and APM-media (3.0×10^6 $cfu.g^{-1}$). An average of 1.2×10^6 $cfu.g^{-1}$ was obtained on the MRS-medium and 7.0×10^4 $cfu.g^{-1}$ on the KCA+TTC-medium. Again, no growth was found on the YELN, Pal-P and YEC-media.

The microbial counts of the Kepi grains packaged in the "methallised oriented polyester film" (MOPET) were similar to those of the grains packaged in the LDPE and OPET. Again, the highest average counts obtained on the KCA+V

Table 3. The average enumeration values ($cfu.g^{-1}$) from normal laboratory produced Kepi grains after three days of activation.

Medium[a]	Enumeration values ($cfu.g^{-1}$)	
MRS	1.4×10^8	$(5.2 \times 10^7 - 2.0 \times 10^8)$
KCA+TTC	1.9×10^8	$(7.4 \times 10^7 - 2.1 \times 10^8)$
KCA+V	3.9×10^4	$(1.2 \times 10^4 - 8.7 \times 10^4)$
APM	1.8×10^8	$(1.3 \times 10^8 - 2.6 \times 10^8)$
YELN	1.9×10^8	$(1.7 \times 10^8 - 2.1 \times 10^8)$
Pal-P	1.3×10^4	$(4.6 \times 10^3 - 2.8 \times 10^4)$
YEC	0	0

[a] The following abbreviations were used: MRS = deMan, Rogosa and Sharpe-medium, KCA = Potassium Carboxymethyl Cellulose Agar, TTC = Triphenyltetrazolium Chloride, APM = Acetobacter Peroxydans Medium, YELN = Yeast Extract Lactate-medium with Naladixic acid, Pal-P = Pal Propiobac-medium, YEC = Yeast Extract Chloramphenical-medium

Table 4. Microbial species isolated from normal laboratory produced Kepi grains after three days of activation.

Microbial species
Lactobacillus fermentum
Lactococcus lactis subsp. *lactis 1*
Lactobacillus brevis 3
Geotrichum candidum

Table 5. The average enumeration values ($cfu.g^{-1}$) from Kepi grains after 10 d of mass production.

Medium[a]	Enumeration values ($cfu.g^{-1}$)	
MRS	5.9×10^7	$(1.8 \times 10^7 - 1.4 \times 10^8)$
KCA+TTC	1.6×10^8	$(1.3 \times 10^8 - 1.9 \times 10^7)$
KCA+V	1.3×10^7	$(1.3 \times 10^6 - 2.0 \times 10^7)$
APM	1.8×10^7	$(2.4 \times 10^6 - 4.0 \times 10^7)$
YELN	8.8×10^7	$(1.3 \times 10^7 - 1.8 \times 10^8)$
Pal-P	2.1×10^4	$(1.7 \times 10^4 - 2.4 \times 10^4)$
YEC	9.2×10^7	$(8.2 \times 10^7 - 1.1 \times 10^8)$

[a] The following abbreviations were used: MRS = deMan, Rogosa and Sharpe-medium, KCA = Potassium Carboxymethyl Cellulose Agar, TTC = Triphenyltetrazolium Chloride, APM = Acetobacter Peroxydans Medium, YELN = Yeast Extract Lactate-medium with Naladixic acid, Pal-P = Pal Propiobac-medium, YEC = Yeast Extract Chloramphenical-medium

Table 6. Microbial species isolated from Kepi grains after 10 d of mass production.

Identification
Lactobacillus plantarum
Lactococcus lactis subsp. lactis 1
Candida lambica
Candida krusei
Geotrichum candidum

Table 7. Microbial growth ($cfu.g^{-1}$) from lyophilised Kepi grains packaged in low density polyethylene film, oriented polyester film and methallised oriented polyester film.

Medium[a]	Sample 1	Sample 2
Low density polyethylene film		
MRS	6.5×10^5	8.0×10^5
KCA+TTC	1.0×10^4	2.1×10^4
KCA+V	2.0×10^6	1.3×10^6
APM	1.1×10^6	3.5×10^6
YELN	0	0
Pal-P	0	0
YEC	0	0
Oriented polyester film		
MRS	2.1×10^5	3.2×10^6
KCA+TTC	6.1×10^4	7.2×10^4
KCA+V	7.2×10^6	5.7×10^6
APM	3.1×10^6	2.9×10^6
YELN	0	0
Pal-P	0	0
YEC	0	0
Methallised oriented polyester film		
MRS	7.7×10^5	1.5×10^5
KCA+TTC	7.3×10^3	9.0×10^3
KCA+V	3.1×10^6	3.2×10^6
APM	2.7×10^6	9.7×10^5
YELN	0	0
Pal-P	0	0
YEC	0	0

[a] The following abbreviations were used: MRS = deMan, Rogosa and Sharpe-medium, KCA = Potassium Carboxymethyl Cellulose Agar, TTC = Triphenyltetrazolium Chloride, V = Vancomycin, APM = Acetobacter Peroxydans Medium, YELN = Yeast Extract Lactate-medium with Naladixic acid, Pal-P = Pal Propiobac-medium, YEC = Yeast Extract Chloramphenicol-medium.

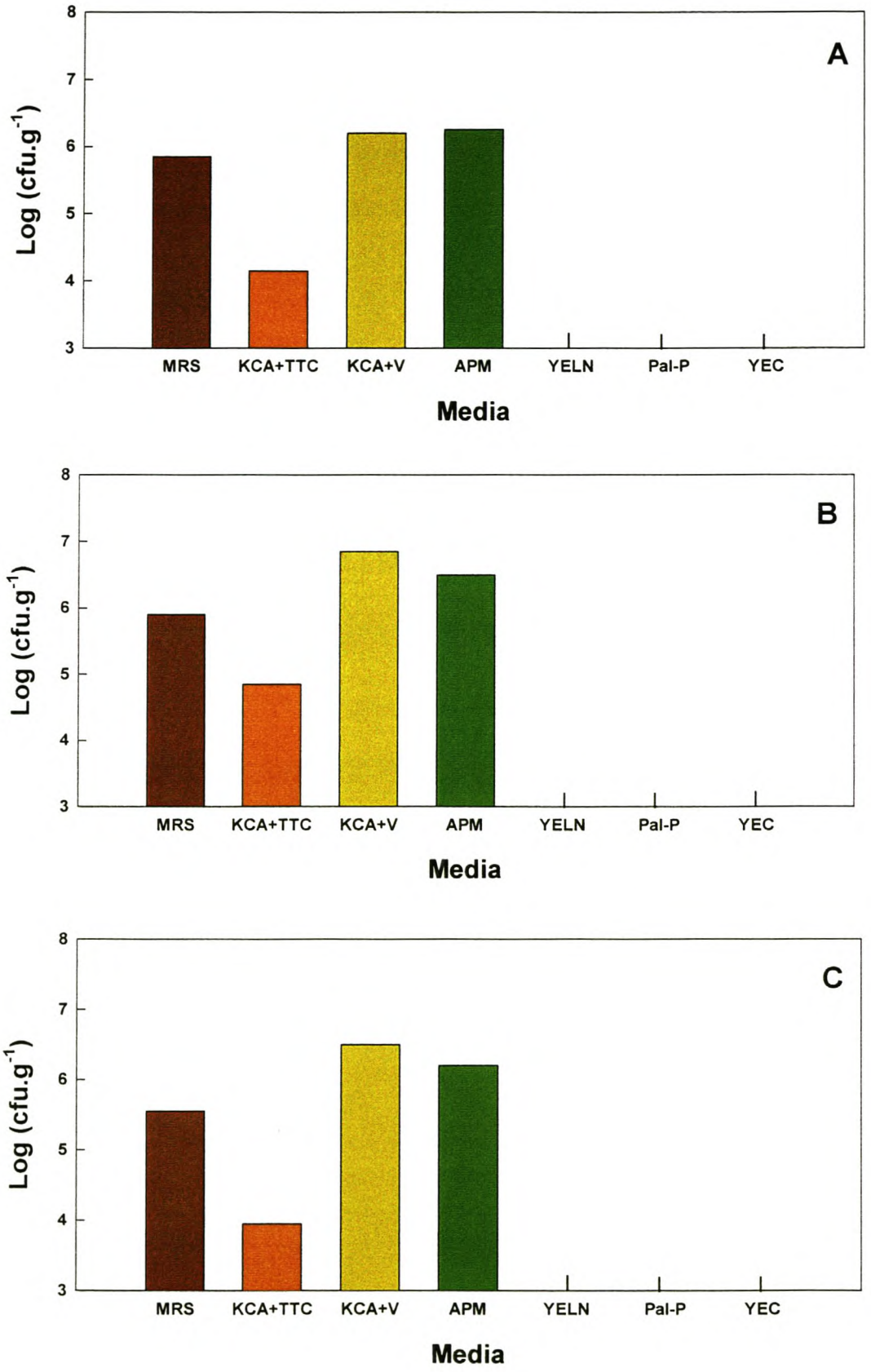

Fig. 1. The average microbial counts obtained from lyophilised Kepi grains packaged in low density polyethylene film (**A**), oriented polyester film (**B**) and methallised polyester film (**C**). No growth was found on the YELN, Pal-P and YEC-media.

(3.2×10^6 cfu.g^{-1}) and APM-media (1.8×10^6 cfu.g^{-1}). An average value of 4.6×10^5 cfu.g^{-1} was obtained on the MRS-medium, while an average count of 7.9×10^3 cfu.g^{-1} was obtained on the KCA+TTC-medium. As was found previously for the other two packaging materials, no growth was observed on the YELN, Pal-P and YEC-media.

Although the average microbial numbers obtained on the various media from the Kepi grains packaged in the different packaging materials were very similar, the average of all the counts obtained from the grains packaged in OPET (2.7×10^6 cfu.g^{-1}) were found to be slightly higher than those obtained from the grains packaged in LDPE (1.2×10^6 cfu.g^{-1}) and MOPET (1.4×10^6 cfu.g^{-1}). These results suggest that the difference in the moisture vapour transmission rates and oxygen permeability figures of the different packaging materials (Table 1) did not strongly influence the microbial counts of the different packaged Kepi grains.

It is also necessary to carefully consider whether more expensive packaging materials (Table 1), in effect, also imply better preservation of the Kepi grains. It was also found that the average enumeration values obtained from the Kepi grains in this study are much lower when compared to the average enumeration values obtained from Kepi grains that were not lyophilised and packaged (Control I) and after 10 d of mass production (Control II) (Tables 3 and 5, respectively). It is possible that this decrease in the microbial counts as a result of the lyophilisation and packaging are only temporary and that a longer period of Kepi grain activation and subsequent Kepi production would lead to an increase in the enumeration values. It is also possible that storage of the packaged grains at room temperature for two months could have been the cause of the decrease in the microbial counts, suggesting that the packaged grains should rather be stored at cooler temperatures or even under vacuum conditions.

Identification of the isolates

The morphological and biochemical characteristics of the strains isolated from the stored grains in the different packaging materials, using the API 50 CHL identification system together with the results of the additional tests performed, are given in Table 8. The identification of the microbes isolated from the packaged Kepi grains stored at room temperature for two months, are listed in Table 9.

Table 8. Characterisation (API 50 CHL) of the LAB isolates from Kepi grains that were lyophilised and packaged in LDPE, OPET and MOPET.

Test	LDPE		OPET				MOPET				
	L1 1KT	L1 1KV	L2 1KT	L2 2KT	L2 1KV	L2 2KV	L3 1MS	L3 1KV	L3 2KV	L3 3KV	L3 1A
Gram stain	+	+	+	+	+	+	+	+	+	+	+
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Catalase	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-
Fermentation of:											
Control	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	+	+	-	-	-	-	-
Ribose	-	-	-	-	+	+	-	-	-	-	+
D-Xylose	-	-	-	-	-	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-
ß Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	-	-	+	+	+	+	+
D-Glucose	+	+	+	+	+	-	+	+	+	+	+
D-Fructose	-	+	+	+	+	+	+	+	+	-	+
D-Mannose	+	+	+	+	-	-	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D-mannoside	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D glucoside	-	-	-	-	-	-	-	-	-	-	-
N Acetyl glucosamine	+	+	+	+	-	-	+	+	-	+	+
Amygdaline	-	-	-	-	-	-	-	-	-	-	-
Arbutine	-	-	-	-	-	-	-	-	-	-	-
Esculine	+	-	-	-	-	-	-	-	-	+	+
Salicine	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	+
Maltose	-	-	-	-	-	-	-	-	-	-	+
Lactose	+	+	+	+	-	+	+	+	+	+	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-
Saccharose	+	+	+	+	-	-	+	+	+	+	-
Trehalose	+	+	+	+	-	-	+	+	+	+	-
Inuline	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-	-	-
Amidon	-	-	-	-	-	-	-	-	-	-	-
Glycogene	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-

Table 8. (continued).

Test	LDPE		OPET				MOPET				
	L1 1KT	L1 1KV	L2 1KT	L2 2KT	L2 1KV	L2 2KV	L3 1MS	L3 1KV	L3 2KV	L3 3KV	L3 1A
ßGentlobiose	-	-	-	-	-	-	-	-	-	-	-
D-Turanose	-	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	+	-	-	-	-	-
2 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-
5 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-

Table 9. Identification results of the LAB from the lyophilised Kepi grains packaged in LDPE, OPET and MOPET, using the API 50 CHL and Rapid ID 32 systems.

Isolate number[a]	Identification	Isolation medium	Identification (%)	Identification acceptability
LDPE				
L1 1KT	*Lb. delbrueckii* subsp. *lactis*	KCA+TTC	42.7	Doubtful
L1 1KV	*Lb. delbrueckii* subsp. *lactis*	KCA+V	91.0	Very good
OPET				
L2 1KT	*Lb. delbrueckii* subsp. *lactis*	KCA+TTC	91.0	Very good
L2 2KT	*Lb. delbrueckii* subsp. *lactis*	KCA+TTC	91.0	Very good
L2 1KV	*Lb. brevis*	KCA+V	76.5	Acceptable
L2 2KV	*Lb. brevis*	KCA+V	93.8	Very good
MOPET				
L3 1MS	*Lb. delbrueckii* subsp. *lactis*	MRS	91.0	Very good
L3 1KV	*Lb. delbrueckii* subsp. *lactis*	KCA+V	91.0	Very good
L3 2KV	*Lb. delbrueckii* subsp. *lactis*	KCA+V	89.5	Acceptable
L3 3KV	*Lb. delbrueckii* subsp. *lactis*	KCA+V	42.7	Doubtful
L3 1A	*Lb. curvatus*	APM	97.4	Very good

[a] **Isolate number:** First two digits – sample number; third digit – isolated colony number; and last two digits – isolation medium (MS = MRS-medium, KT = KCA+TTC-medium, KV = KCA+V-medium, A = APM-medium).

The microbes isolated and identified from the Kepi grains packaged in LDPE included only strains of *Lb. delbrueckii* subsp. *lactis*. Kepi grains packaged in OPET contained *Lb. delbrueckii* subsp. *lactis* and *Lb. brevis*, while grains packaged in MOPET contained *Lb. delbrueckii* subsp. *lactis* and *Lb. curvatus*. No propionibacteria, AAB or yeasts were isolated from any of the packaged Kepi grains.

The lyophilised, packaged Kepi grains included a much smaller diversity of microbes (only two species at most) compared to that of the Kepi grains in Control I, as well as Control II (Tables 4 and 6, respectively). Different microbes were also isolated compared to the microbes isolated from the Kepi grains in Controls I and II. Although the microbial differences is most likely due to the lyophilisation and/or packaging of the grains, it might also be because the packaged grains were stored at room temperature, in which case storage at cooler temperatures might be advisable. Furthermore, it is possible that a longer period of activation and subsequent Kepi production would lead to an increase in the number of microbial species present in the grains. As with the microbial counts obtained from the packaged Kepi grains, the impact of the moisture vapour transmission rates and oxygen permeability figures of the different packaging materials (Table 1) on the microbial community of the different grains, seems minimal. Again, it should be considered whether more expensive materials (Table 1) also imply better preservation of the Kepi grains.

It is interesting to note that yeasts were not isolated from either the lyophilised, packaged Kepi grains or from the Kepi grains that were not lyophilised or packaged and only activated for three days (Control I). The absence of yeasts in this study can, therefore, not solely be attributed to the lyophilisation or the packaging of the grains. It is possible that the yeasts are only present in very low concentrations at this early stage of Kepi production or that they may not yet be active. Furthermore, Bottazzi & Bianchi (1980) reported that yeasts normally dominated in the centre of the grains, which would, at low concentrations, make them difficult to isolate.

In this study, the selectivity of the media used to isolate LAB (MRS, KCA+TTC and KCA+V) were found not to be very specific as *Lb. delbrueckii* subsp. *lactis* was found to grow on all three of the media (Table 10). *Lactobacillus brevis* was isolated from the APM-medium, while *Lb. curvatus* was isolated from

Table 10. The microbial structure of the Kepi grains packaged in LDPE, OPET and MOPET, as depicted by the growth medium.

Species	Number of isolates [a]						
	MRS	KCA+ TTC	KCA+ V	APM	YELN	Pal-P	YEC
LDPE							
Lb. delbrueckii subsp. *lactis*	-	1	1	-	-	-	-
OPET							
Lb. delbrueckii subsp. *lactis*	-	2	-	-	-	-	-
Lb. brevis	-	-	2	-	-	-	-
MOPET							
Lb. delbrueckii subsp. *lactis*	1	-	3	-	-	-	-
Lb. curvatus	-	-	-	1	-	-	-

[a] The following abbreviations were used: MRS = deMan, Rogosa and Sharpe-medium, KCA = Potassium Carboxymethyl Cellulose Agar, TTC = Triphenyltetrazolium Chloride, V = Vancomycin, APM = Acetobacter Peroxydans Medium, YELN = Yeast Extract Lactate-medium with Naladixic acid, Pal-P = Pal Propiobac-medium, YEC = Yeast Extract Chloramphenicol-medium

the KCA+V-medium. This lack of selectivity was also observed in Chapter 3 of this thesis where the microbial content of Kepi grains were isolated using the same media. It must be emphasised that careful consideration should be given before making conclusions about the microbial content of a fermentation environment based on media selectivity alone.

Few studies have been reported in the literature on the lyophilisation or packaging of Kepi grains. Ohara *et al.* (1998) studied lyophilised Kepi grains from Georgia, Russia. They found *Weisella confusa* to be the predominant microbe, but ascribed its presence to be the result of contamination during the activation of the lyophilised Kepi grains. They furthermore isolated and identified *Lb. kefir* and *Lb. kefiranofaciens*, with the possibility that two isolates identified as *Lb. kefiranofaciens* might be *Lb. acidophilus* and one isolate might be *Lb. delbrueckii* subsp. *lactis*. In this study, *Lb. delbrueckii* subsp. *lactis* was isolated from the lyophilised Kepi grains packaged in all three packaging films. The absence of certain microbes previously isolated from Georgian Kepi grains (*Lactococcus lactis* and *Leuconostoc mesenteroides*) was reportedly due to culture conditions prior to the lyophilisation of the grains (Ohara *et al.*,1998).

Distribution frequency

The Harrison Disc Method (Harrigan, 1998) used for the random statistical selection of representative colonies, allowed for the calculation of the percentage distribution of the microbes present in the Kepi grains packaged in the different packaging materials. The distribution frequencies of the prevalent LAB isolated from the Kepi grains packaged in LDPE, OPET and MOPET are illustrated in Fig. 2A - C. The microbial species, as depicted in these figures, represent the dominant (numerically dominant) species that were isolated from the Kepi grains in each case.

The fact that *Lb. delbrueckii* subsp. *lactis* was isolated from the Kepi grains in all three packaging materials suggests that this species is not as easily influenced by factors such as the moisture vapour transmission rates and oxygen permeability figures of the different packaging materials. Figures 2B and 2C, furthermore, clearly depict that although two species were isolated from grains packaged in both OPET and MOPET, *Lb. delbrueckii* subsp. *lactis* represented only 1.0% of the population in the grains packaged in OPET, in contrast to 51.5%

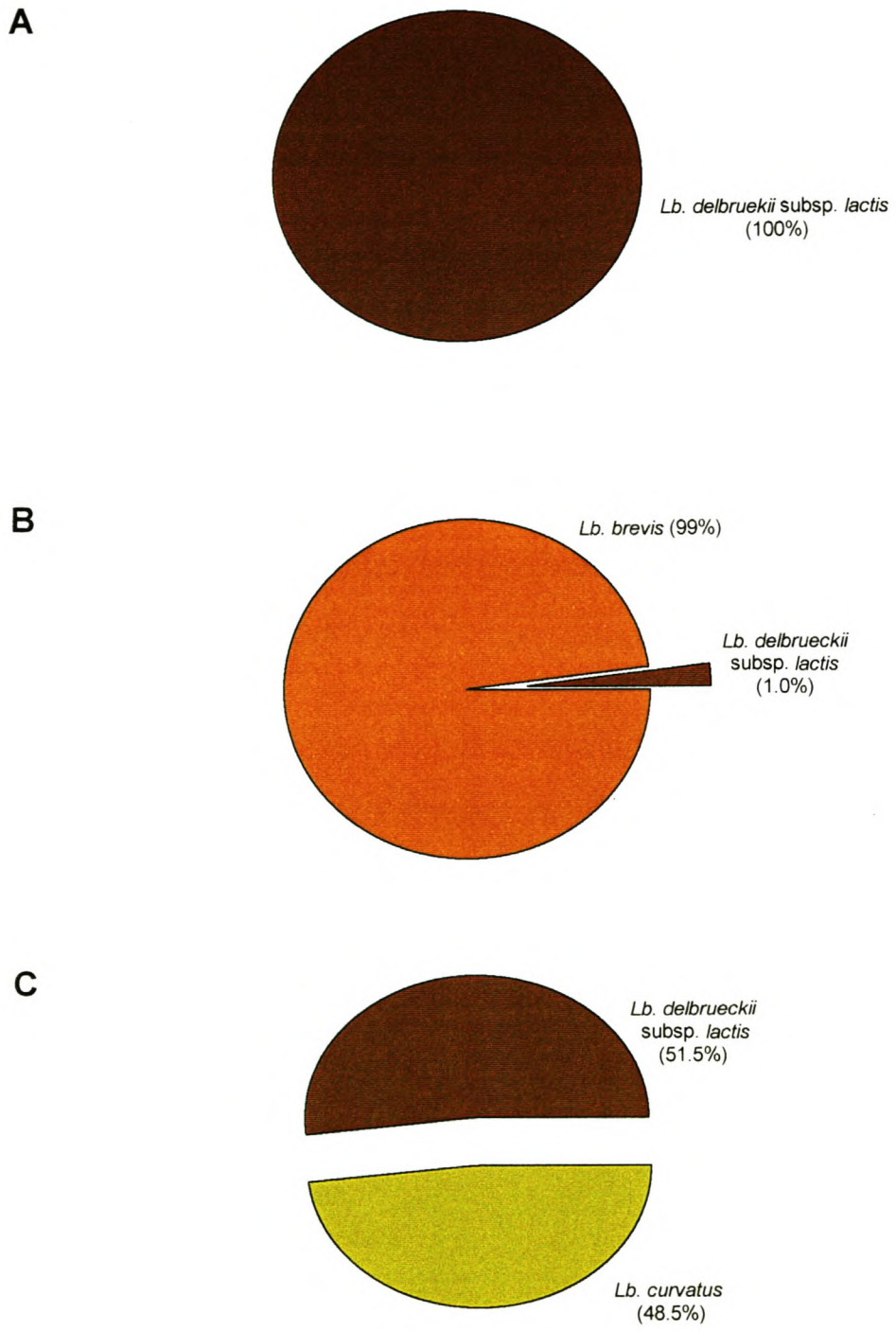

Fig. 2. The distribution frequency of the prevalent isolates from lyophilised Kepi grains packaged in low density polyethylene film (**A**), oriented polyester film (**B**) and methallised oriented polyester film (**C**).

in the grains packaged in MOPET. *Lactobacillis brevis* made up 99% of the population in the grains packaged in OPET, while *Lb. curvatus* was present at 48.5% in the grains packaged in MOPET. It might be that, when Kepi grains are lyophilised and/or packaged, a longer activation time is needed for certain microbes to develop, suggesting that Kepi should also be produced from the dried grains before definite conclusions are made about the impact of packaging materials on Kepi beverage quality.

Conclusion

In this study the data clearly showed that lyophilisation and packaging of Kepi grains resulted in a reduction of the microbial numbers, as well as the microbial species, compared to Kepi grains that were not lyophilised or packaged (Controls I and II). Although the microbes isolated from the Kepi grains packaged in LDPE, OPET and MOPET did not vary greatly from each other, the microbial species isolated from the packaged Kepi grains were very different from the microbial species isolated from Kepi grains in Controls I and II. It is, therefore, clear that the lyophilisation and packaging of Kepi grains do have a definite impact on the microbial community of Kepi grains. However, it is recommended that Kepi be produced for a longer period to determine whether the packaged Kepi grains produce a Kepi beverage with organoleptic properties similar to Kepi produced by normal grains. The successful packaging of Kepi grains will undoubtedly greatly enhance future possibilities for the commercial marketing of Kepi and Kepi grains.

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CHAPTER 5

ENRICHMENT OF KEPI WITH PROPIONIBACTERIA

Abstract

Kepi is a fermented milk beverage that is reported to possess various health properties and is generally considered to have a high nutritional value (Libudzisz *et al.*, 1990). However, the vitamin B_{12} concentration of Kepi is known to decrease during the fermentation process. Since vitamin B_{12} can be produced by propionibacteria (PAB), the enrichment of Kepi grains with PAB was investigated in this study. A polymerase chain reaction (PCR) method for the rapid identification of PAB was developed and this method, together with the use of the selective medium, Pal Propiobac, was used to identify PAB in the Kepi beverage and the Kepi grains. PAB were identified as a natural part of Kepi grains that were sampled from certain stages of Kepi production, using the Pal Propiobac-medium. However, PCR results of the same grain samples and subsequently the microbial colonies on the Pal Propiobac-medium, were negative for the presence of PAB. It was not possible to enrich the Kepi grains successfully during the mass production of the Kepi grains. However, it was determined that various factors possibly inhibited the PAB to become part of the microbial community of the Kepi grains. These factors included the inhibition of PAB by certain lactic acid bacteria (LAB) in the Kepi grains (*Lactobacillus plantarum*), the inability of certain LAB (*Lactococcus lactis* subsp. *lactis*) in the grains to remove possible inhibiting factors and also the inoculation concentration of propionibacteria in Kepi.

Introduction

Kepi is a slightly carbonated and acidic fermented product that is made by fermenting milk with a complex mixture of bacteria and yeasts, present in the form of grains (Roginsky, 1988). As a fermented milk beverage, Kepi is considered to be very nutritious (Saloff-Coste, 1999). However, it has been reported that although fermented milk products usually have a nutrient composition that is very similar to that of milk (Steinkraus, 1996), the fermentation process does lead to a decrease in

the concentration of certain vitamins (Gurr, 1987). A study done by Karlin (1966) has shown that the content of B vitamins, including vitamin B_{12}, in Kepi decreased by as much as 95% during the lactic acid fermentation of milk. However, Karlin (1966) also reported that Kepi enriched with propionibacteria (PAB) contained higher amounts of vitamin B_1, B_2, B_6, niacin, B_{12}, pantothenate, folic and folinic acid.

The genus *Propionibacterium* can be divided into two main ecological groups: the cutaneous and the "classical" or dairy propionibacteria. The "classical" group are important starter organisms in many dairy fermentations (Lyon & Glatz, 1995). Traditionally, PAB are used for their ability to convert lactate to propionate, acetate and CO_2, where the CO_2 is responsible for the formation of the characteristic eyes in Swiss-type cheese (Suomalainen & Mäyrä-Mäkinen, 1998). However, the ability of PAB to produce vitamin B_{12} opens up potential new roles for them in the production of medicine, livestock supplementation and the enrichment of dairy products such as Kepi (Vorobjeva, 1999).

Černá & Hrabová (1977) achieved a 60 fold increase in the vitamin B_{12} levels of Kepi through enrichment with PAB, while Mann (1979) reported a 28 fold increase in the vitamin B_{12} concentration of Kepi enriched with a "*Propionibacterium shermanii*" culture. Polish researchers also reported successful vitamin B_{12} enrichment of Kepi using a culture of "*P. petersonii*" T-112 in combination with Kepi grains (Mann, 1979). Beside the increased vitamin B_{12} levels in Kepi, it has been reported that the addition of PAB to the lactic acid bacterial starters (LAB) that are used in the fermentation of vegetables also led to increased vitamin B_{12} levels in these products (Babuchowski *et al.*, 1998).

The aim of this study was to determine whether Kepi grains could be enriched with a *P. freudenreichii* subsp. *freudenreichii* strain and if the added PAB could be detected in the Kepi beverage and Kepi grains using a polymerase chain reaction (PCR) method.

Materials and methods

Mass production

The mass production of the Kepi grains was done according to the method developed by Schoevers (2000) and patented (SA Patent 2000/1896). The procedure involved the addition 2% (w/v) yeast extract (Biolab) and 0.5% (w/v) urea

(Biolab) to 400 ml of double pasteurised full cream milk. Forty grams of activated Kepi grains were then added to the pasteurised milk mixture and the containers incubated at 25°C in a shake waterbath. The grains were sieved out and placed in a new milk mixture containing the additional yeast extract and urea, every 24 h.

Homogenisation of Kepi grains

Ten gram of Kepi grains were homogenised in 90 ml of sterile saline solution (SSS) (0.85% (w/v) NaCl) using a Stomacher (BagMixer, Interscience, France). The grains were homogenised for 15 min.

Strains and test samples

All the PAB strains used in this study were obtained from the University of Stellenbosch Food Science Culture Collection (USFSCC), Stellenbosch, South Africa. The PAB reference strains included *P. acidopropionii*, *P. thoenii*, *P. shermanii* and *P. freudenreichii* subsp. *freudenreichii*, each representing one of the four main groups based on ribotyping (Table 1) (Riedel *et al.*, 1994). These four strains were first cultured in YEL-medium, incubated anaerobically at 30°C for 5 d and then maintained on YEL-agar. The YEL-medium consisted of ($g.l^{-1}$): yeast extract (Biolab) 5.0; sodium lactate (Saarchem) (60% v/v) 20.0; and Tween 80 (Merck) 1.0 ml. For the YEL-agar, 15 $g.l^{-1}$ agar (Biolab) was added. The pH of the YEL-medium and YEL-agar were adjusted to pH 7.2 before sterilisation using a 1M NaOH solution.

Test samples were also taken from a Swiss-type cheese and from UASB bioreactor granules (Joubert, 2001), where it was known that PAB were present.

Control samples

Three control samples were prepared for the identification of PAB in Kepi using the PCR method. The samples included 1 ml SSS (water control), 1 ml milk (milk control) and 1 ml Kepi (Kepi control), all inoculated with 1 x 10^8 $cfu.ml^{-1}$ of a *P. freudenreichii* subsp. *freudenreichii* strain (Table 1). The control studies were done to rule out the possibility of inhibiting factors present in milk or Kepi that may possibly interfere with the amplification process.

DNA isolation

DNA from microbes present in Kepi grains, as found in Chapter 3 of this thesis (Table 2), was isolated according to the procedure of Van Elsas *et al.* (1997). One to two Kepi grains, 0.6 g sterile glassbeads, 800 µl of a 120 mM Na-phosphate buffer (120 mM NaH_2PO_4:120 mM Na_2HPO_4 (9:1), pH 8.0), 700 µl phenol and 100 µl 20% sodium dodecyl sulphate (SDS) were vortexed for 1 min and incubated at 60°C for 20 min. The vortex and incubation steps were repeated twice, after which the tubes were centrifuged at 15 000 x g for 5 min. A phenol extraction step was followed by chloroform:phenol:isoamylalcohol (24:25:1) extractions. The DNA was precipitated on ice for 30 min with 0.1 volume 3 M sodium acetate (pH 5.5) and 0.6 volume isopropanol and then centrifuged at 134 000 x g for 10 min. Once the supernatant was removed, the pellet was washed with 70% ethanol, dried and redisolved in TE buffer (10mM Tris, 1mM EDTA; pH 8.0). The extracted DNA was separated on a 1% agarose gel, stained with ethidium bromide and visualised under UV light.

PCR

Specific PCR primers (Prop 1 and Prop 2) were used in this study. These primers were designed based on aligned 16S rRNA gene sequences of the specific PAB reference strains as given in Table 1 (Riedel *et al.*, 1998).

The 50 µl PCR reactions contained 1U of *Taq* DNA polymerase buffer (Bioline), 2mM $MgCl_2$, 1mM dNTPs (Promega), 0.5 µl of the Prop 1 primer (5'-GATACGGGTTGACTTGAGG-3') and 0.5 µl of the Prop 2 primer (5'-GTAATCGCAGATCAGCAACG C-3'). To each reaction mixture, 1 µl of cells or 10 µl of extracted DNA was added. The PCR reactions were performed on a Mastercycler (Eppendorf) with the reaction conditions as follows: initial denaturation was performed at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 35 s, annealing at 60°C for 1 min, and chain elongation at 72°C for 1 min. A final chain elongation step was performed at 72°C for 10 min, followed by the cooling of the products to 25°C. The PCR reaction products were separated on a 1% agarose gel in 0.5 TBE electrophoresis buffer. The gel was stained with ethidium bromide and visualised under UV light.

Table 1. *Propionibacterium* strains used in this study.

USFSCC number [a]	Species	Groups based on ribotyping[b]
424	*P. acidopropionii*	A
419	*P. thoenii*	T
80	*P. jensenii*	J
423	*P. freudenreichii* subsp. *freudenreichii*	F

[a] University of Stellenbosch Food Science Culture Collection, Stellenbosch, South Africa

[b] Riedel *et al.* (1996)

Table 2. The microbial composition of Kepi grains at various stages of Kepi and Kepi grain production (see Chapter 3 of this thesis).

Control I – After three days of Kepi grain activation	Control II – After 10 d of mass production	Control III – After 30 d of normal Kepi production
Lb. fermentum	-	*Lb. fermentum*
Lb. brevis 3	-	-
Lc. lactis subsp. *lactis 1*	*Lc. lactis* subsp. *lactis 1*	-
-	-	*Lb. delbrueckii* subsp. *delbrueckii*
-	-	*Leuc. mesenteroides* subsp. *cremoris*
-	-	*C. kefyr*
-	*Lb. plantarum*	-
-	*C. lambica*	-
-	*C. krusei*	-

Identification of PAB in Kepi and Kepi grains (not PAB enriched)

Samples of Kepi beverage and grains were analysed in triplicate for the presence of PAB to determine whether PAB might be a natural component of the grains used in this study and if so, could they be detected easily. The Kepi beverage and grains that were analysed, were obtained from Kepi grains subjected to various stages of Kepi production, described in Chapter 3 of this thesis, as Control I (after three days of activation), Control II (after 10 d of mass production) and Control III (after 30 d of normal Kepi production). The Kepi beverage and Kepi grains were analysed for the presence of PAB using the selective medium, Pal Propiobac (Thierry & Madec, 1995), as well as using the PCR method that was designed in this study for the rapid identification of PAB. A positive result for PAB on the Pal Propiobac-medium was indicated by a colour change in the medium from purple to yellow.

Kepi enrichment with PAB

Propionibacterium freudenreichii subsp. *freudenreichii* (strain 423) was grown at 30°C in YEL-medium to an OD of 0.65 at 540 nm (1 x 10^6 cfu.ml^{-1}). The cells were centrifuged at 5 000 x g for 10 min, after which the cells were washed three times with 10 ml SSS. The cell pellet was dissolved in 1 ml SSS and added to the Kepi grains during the mass production. This step was repeated daily for 10 consecutive days during the mass production of the grains. The Kepi beverage and grains produced during the 10 d of mass production with the PAB enrichment were analysed for the presence of PAB on days 0, 2, 4, 6, 8 and 10 by using the PCR detection method and also by plating 0.1 ml decimal dilutions of the Kepi beverage and the homogenised Kepi grain samples on the selective Pal Propiobac-medium.

Results and discussion

PCR of the PAB reference strains, test and control samples

The targeted sequence of the 16S ribosomal DNA gene of all four the PAB reference strains was successfully amplified using the primers Prop 1 and Prop 2. The amplified fragments were all 720 bp in size (Fig. 1).

PCR reactions of a homogenised sample taken from the Swiss-type cheese known to contain PAB, as well as from a sample taken from UASB bioreactor

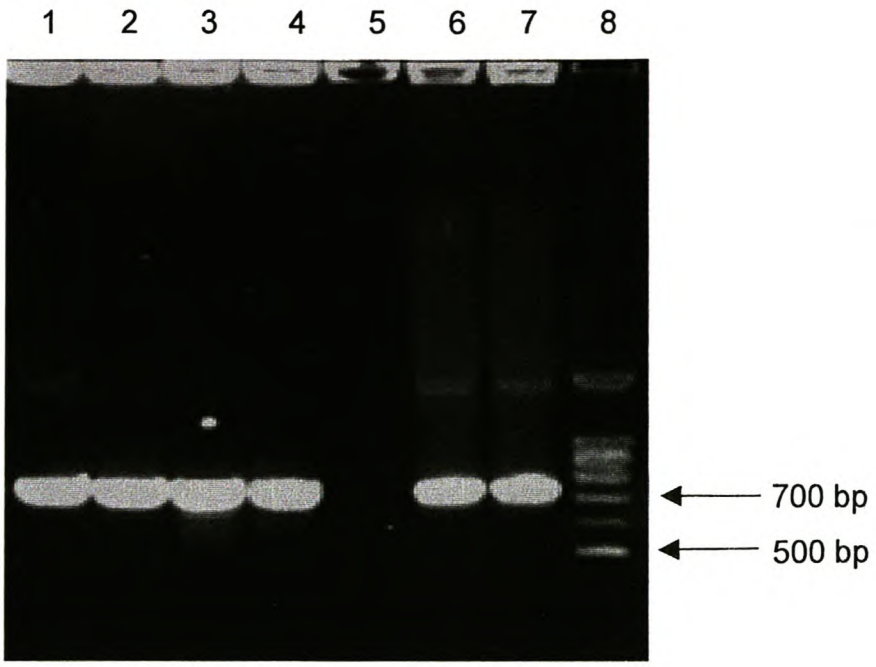

Figure 1. A 1% (w/v) agarose gel showing the PCR fragments generated from 16S ribosomal DNA of the selected propionibacteria. Lanes: 1, *P. acidopropionii*; 2, *P. freudenreichii* subsp. *freudenreichii*; 3, *P. jensenii*; 4, *P. thoenii*; 5, water control; 6, *Propionibacterium* species present in Swiss type cheese; 7, *Propionibacterium* species present in UASB bioreactor granule; 8, 100 bp ladder.

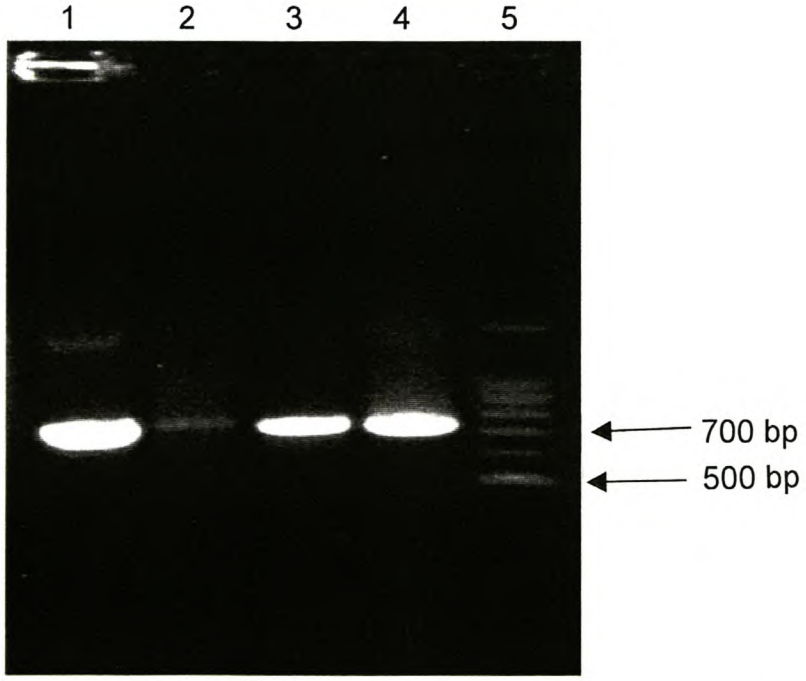

Figure 2. An 1% (w/v) agarose gel showing the PCR fragments generated from 16S ribosomal DNA of propionibacteria in selected samples. Lanes: 1, positive control; 2, Kepi with *P. freudenreichii* subsp. *freudenreichii*; 3, milk with *P. freudenreichii* subsp. *freudenreichii*; 4, water with *P. freudenreichii* subsp. *freudenreichii*; 5, 100 bp ladder.

granules known to contain PAB (Joubert, 2001), were also carried out. Bands of 720 bp were obtained from the cheese and bioreactor granule samples (Fig. 1), resulting in a positive identification for the presence of PAB and supporting the use of the PCR method for the identification of PAB in the Kepi beverage and grains.

The PCR products generated from all the control samples (water control, milk control and Kepi control) also produced bands that were 720 bp in size (Fig. 2), indicating the successful amplification of the targeted sequence of the 16S ribosomal DNA gene of the *P. freudenreichii* subsp. *freudenreichii* strain added to each control. The positive results suggested that there were no factors in the milk or Kepi that would inhibit the PCR detection of PAB in these two products.

Identification of PAB in Kepi and Kepi grains (not PAB enriched)

Control I – After three days of activation: No typical yellow colonies were found on the selective Pal Propiobac-medium from either triplicate Kepi beverage samples or triplicate Kepi grain samples that were tested after three days of activation. Negative PCR results were also obtained when the Kepi beverage, as well as the Kepi grain samples, were tested for the presence of PAB. It was thus concluded that there were no PAB present in the Kepi beverage or grains after three days of activation.

Control II – After 10 d of mass production: After 10 d of mass production yellow colonies were visible on the Pal Propiobac-medium from both the Kepi beverage and Kepi grain samples, resulting in a positive identification for PAB as described by Thierry & Madec (1995).

However, PAB were not detected in the Kepi beverage or the Kepi grain samples when the PCR method was used. DNA isolation from the Kepi grain samples also produced negative PCR results. The actual yellow colonies obtained on the Pal Propiobac-medium were then tested using the PCR method and, again, negative results for PAB were obtained. Negative results for PAB were also obtained by gas chromatography (Varian 3700 GC) analysis of the metabolites produced by the microbial colonies on the Pal Propiobac-medium. It was thus concluded that there were no PAB present in the Kepi beverage or grains after 10 d of mass production.

Control III – After 30 d of Kepi production: The results obtained from the samples of Kepi beverage and grains analysed for the presence of PAB after 30 d of

Kepi production were the same as found for the samples analysed after 10 d of mass production (Control II). Although positive results for the presence of PAB in the Kepi and Kepi grain samples were obtained using the selective Pal Propiobac-medium according to the criteria described by Thierry & Madec (1995), PCR results obtained with the samples, were negative. The DNA isolation of the Kepi grain samples also produced negative PCR results, as did the colonies obtained on the Pal Propiobac-medium. Negative results for PAB were also obtained by gas chromatography analysis of the metabolites produced by the microbial colonies on the Pal Propiobac-medium. These colonies were then characterised using the API 50 CHL system and identified as *Lb. delbrueckii* subsp. *delbrueckii*.

Negative PCR results for the presence of PAB was obtained from the Kepi beverage, Kepi grains, the DNA isolated from the grains, as well as the colonies isolated on the Pal Propiobac-medium. Since the PCR method was successful in the detection of PAB using reference PAB strains (Table 1) and also samples of cheese and bioreactor granules known to contain PAB, it was concluded that there were no PAB naturally present in the Kepi beverage or grains that were analysed in this study.

Kepi enrichment with PAB

In this study, where Kepi was enriched with PAB, the triplicate Kepi beverage and grain samples tested on days 0, 2, 4, 6, 8 and 10 of the PAB enrichment, were found to be negative with the PCR method. Furthermore, no yellow colonies were also found on the Pal Propiobac-medium, resulting in a negative identification for PAB when the method described by Thierry & Madec (1995) was used. These results suggest that the PAB might not survive under the selective pressures of the Kepi environment, which is quite unusual for bacteria known for their wide presence in dairy products, especially such as milk and cheese.

Piveteau *et al.* (2000) reported that an initial level of at least 10^6 cells per ml was necessary for propionibacteria to grow in milk. This was the concentration used for the enrichment of the Kepi in this study, but Piveteau *et al.* (2000) reported that even at these levels the growth of propionibacteria in whey was inhibited and decreased due to the presence of unknown inhibitory substance(s). In their study, the growth of *Lb. helveticus* in milk resulted in the destruction of the inhibitor(s). Several other LAB strains, including *Lc. lactis* subsp. *lactis*, however, failed to

remove the substance(s). It should be noted that this species was also isolated from Kepi grains after 10 d of mass production (Table 2), at which time the enrichment of Kepi with *P. freudenreichii* subsp. *freudenreichii* was performed. It is, therefore, possible that an inhibitory substance, not removed by the LAB present in the Kepi grains during mass production, might have kept the PAB from becoming a part of the microbial consortium of Kepi. Propionibacteria are also reported to be inhibited by *Lb. plantarum* (Ouwehand, 1998), which was also found to be present in the Kepi grains after 10 d of mass production (Table 2).

The positive PCR results obtained with the Kepi control, as discussed in the previous section, (1 ml Kepi inoculated with 1 x 10^8 cfu.ml^{-1} PAB) is possibly due to the fact that a much higher PAB concentration was present in the Kepi control than in the Kepi that was enriched with PAB. It is, therefore, possible that a higher PAB inoculum concentration for the enrichment of Kepi is necessary in order for the PCR method, as used in this study, to detect the added PAB. If PAB is then detected in the Kepi beverage, but still not in the Kepi grains, it should be determined whether PAB has, in fact, been incorporated into the grains and not been prevented in some way in becoming part of the microbial community of Kepi grains.

Conclusion

A rapid PCR based detection method for PAB was successfully developed and tested with PAB reference strains, as well as PAB strains present in Swiss-type cheese and UASB bioreactor granule samples. The use of molecular methods, such as PCR, for the identification of microbes in food products have proven to be very successful. These methods are very accurate and time-efficient and can not only be used to identify spoilage microbes in food products, but can also be used to determine whether the enrichment of a product such as Kepi with PAB was successful.

The fact that a positive result for the presence of PAB was obtained from the Kepi control (1 ml of Kepi inoculated with 1 x 10^8 cfu.ml^{-1} PAB) using the PCR method, confirms that it is possible to detect PAB in Kepi using this method. However, the PCR results obtained in this study suggest that a higher PAB inoculum concentration than what was used (1 x 10^6 cfu.ml^{-1}), is necessary for the detection of PAB in Kepi using the PCR method.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Background

It is known that various environmental factors play a role in the development of the many fermented milk products known today (Vedamuthu, 1982). Kepi is such a fermented milk product. However, the unique feature of Kepi is the use of small, irregularly shaped grains as starter to produce the beverage. These grains are composed of proteins and polysaccharides in which lactic acid bacteria (LAB) and yeasts are embedded (Pintado *et al.*, 1996). It has been shown that the microbial community of Kepi grains is influenced by environmental factors such as the method by which Kepi is produced, the milk used, the origin of the grains and the storage conditions of the grains (Garrote *et al.*, 1997; Pintado *et al.*, 1996). This variation in the microbial community of the Kepi grains makes it extremely difficult to determine the exact microbial composition of the grains. Since the microbial community will change, depending on the prevailing environmental conditions, it makes more sense to determine the impact of the individual factors on the microbial community of the grains than trying to establish the exact microbial composition of the grains. In this study the impact of the different stages of Kepi production, grain origin, and lyophilisation and packaging of the grains on the microbial community structure of the grains, were determined. The enrichment of Kepi grains with propionibacteria and the detection of propionibacteria in the Kepi beverage and grains with a polymerase chain reaction (PCR) technique were also evaluated.

Impact of environmental changes on the microbial community of Kepi grains

The impact of environmental changes on the microbial community of laboratory produced grains were evaluated in this study. The changes included the method of Kepi production, the origin of the Kepi grains and the lyophilisation and packaging of the grains using three different packaging materials.

Method of Kepi production: The impact of the different stages in the method of Kepi production (activation, mass production and normal Kepi production), was clearly

shown by the gradual decrease of the microbial counts after each stage and was especially evident when the microbes isolated at each stage were evaluated. The combination of microbes present in the Kepi grains after activation, mass cultivation and normal Kepi production was very different. *Lactococcus lactis* subsp. *lactis 1* (isolated after activation and mass cultivation) and *Lactobacillus fermentum* (isolated after activation and normal Kepi production) were the only species isolated at more than one stage of Kepi production. Other species isolated included combinations of *Lb. brevis 3*, *Lb. plantarum*, *Candida lambica*, *C. krusei*, *Lb. delbrueckii* subsp. *delbrueckii*, *Lb. mesenteroides* subsp. *cremoris* and *C. kefyr*. However, at all three stages of Kepi production the isolated LAB, including strains from the genera *Lactococcus* and *Lactobacillus*, were present at a higher concentration than the isolated yeasts. It is clear that the different stages of Kepi production did not influence this relationship between the LAB and yeasts, indicating the importance of this relationship in the production of Kepi.

After three days of activation, it was found that no yeasts were present in the grains. As soon as yeast extract and urea were added to the milk as part of the mass cultivating process and stress conditions were applied to the grains, yeasts were isolated from the grains. The presence of yeasts and the increased yeast counts after the addition of yeast extract and urea as part of the mass cultivation process suggests that the addition or removal of specific compounds can be used to manipulate the microbial combination in the grains. Furthermore, two yeast species, *Candida lambica* and *C. krusei*, isolated after 10 d of mass production have never before, as far as is known, been reported to be part of the microbial community of Kepi grains. The isolation of these two yeast species confirms that the method by which Kepi is produced can impact the microbial community of Kepi grains to such an extent that microbes that have not been reported to be part of the microbial community of Kepi grains, are suddenly found.

When the normal method of Kepi production was resumed after mass production, the combination of the microbes present in the grains changed again and was found to be different than that found after the activation step, as well as after the mass production of the grains. This change in the microbial composition further confirms that the method of Kepi production has a definite impact on the microbial community of Kepi grains. It is inevitable that the presence of different microbial populations in the grains will influence the quality of the Kepi beverage that is

produced. This variation in microbial population after each stage suggests that the method of Kepi production used should be one that establishes a microbial community in the Kepi grains that will produce a safe Kepi beverage of optimum quality with a good shelf-life. A standardised method for the production of Kepi will possibly lead to less variation in the microbial population of the grains and subsequently to a quality beverage.

Grain origin: The impact of grain origin on the microbial community of Kepi grains was confirmed by studying the microbial communities of grain samples obtained from eight different Southern African sources. The impact of the grain sources could be seen in the varying microbial counts obtained from the different grain samples and also in the varying microbial combinations that were isolated from each of them. This variation in the microbial community suggests that, even if all the other factors that influence the microbial community of Kepi grains could be either standardised or excluded, the origin of the grains would still lead to variation in the Kepi grain community. Kepi beverages and grains prepared and used at different sources will propably never consist of just one specific set of microbes.

Lyophilisation and packaging: The microbial community of the laboratory produced Kepi grains that were lyophilised and packaged, resulted in a considerable decrease in the isolated microbial counts, as well as a decrease in the species diversity of the grains. More importantly, the microbial species isolated from the lyophilised and packaged grains after activation were different from those isolated from the normal laboratory produced grains after activation. Although no yeasts were isolated from either the packaged or normal Kepi grains, only *Lactobacillus* strains were isolated from the packaged grains. This difference in the microbial types confirms that the lyophilisation and the packaging of Kepi grains impacts the microbial community of Kepi grains and this must be taken into consideration during the production of Kepi with these grains. A longer activation period might be needed for optimum microbial growth.

In contrast to the above differences, the microbial counts and specific microbes isolated from the grains packaged in the different packaging materials, were very similar. The similar results suggest that the characteristics of the individual packaging materials did not strongly influence the microbial community of the Kepi

grains. It can thus be concluded that packaging materials for Kepi grains should rather be evaluated on the quality of Kepi produced with the packaged grains than by the specific characteristics of the packaging materials.

Enrichment of Kepi grains with propionibacteria

Kepi is a fermented milk beverage with exciting marketing possibilities as it possesses various unique qualities including a very simplistic and economical production technology, good shelf-life and a wide range of health benefits. The successful enrichment of Kepi grains with propionibacteria could result in a product with a higher vitamin B_{12} concentration, contributing positively to the unique Kepi qualities.

In this study, the enrichment of Kepi grains with propionibacteria was evaluated and a polymerase chain reaction (PCR) method for the rapid detection of propionibacteria in the Kepi beverage and grains, was developed. The targeted sequence of the 16S rRNA of four reference propionibacteria strains, as well as strains of propionibacteria present in a Swiss-type cheese and in UASB bioreactor granules were successfully amplified using the PCR technique. Propionibacteria added to 1 ml Kepi at a concentration of 1×10^8 cfu.ml^{-1} (labelled the Kepi control), were also detected using this method.

The enrichment of Kepi grains was then evaluated by adding a *Propionibacterium freudenreichii* subsp. *freudenreichii* strain to the Kepi during the mass cultivation of the grains, at a concentration of 1×10^6 cfu.ml^{-1}. The propionibacteria that were added to the Kepi could not be detected with the polymerase chain reaction (PCR) technique used in this study. However, the positive PCR results obtained from the Kepi control suggests that a propionibacteria inoculum concentration higher than 1×10^6 cfu.ml^{-1} is necessary during the enrichment process for the PCR detection of propionibacteria in the Kepi beverage.

It was also determined that there are many factors that play a role in the successful incorporation of propionibacteria in Kepi grains. These factors include the size of the inoculation concentration, inhibitory substance(s) not removed by the LAB present in the Kepi grains and the inhibition of propionibacteria by certain LAB in the Kepi grains. Thus it was concluded that if it is found that Kepi grains do not contain any propionibacteria after enrichment with a *Propionibacterium* strain, not only the

method by which this is determined should be evaluated, but also whether propionibacteria can actually be incorporated into Kepi grains.

Still, the PCR method has proven to be a successful alternative to the traditional identification methods used for propionibacteria. The effectiveness and accuracy of molecular methods such as PCR, make these techniques invaluable in the rapid identification of microbes.

Concluding remarks

The data from this study clearly showed that the microbial community of Kepi grains consists of various LAB and yeasts that exist in a complex symbiotic relationship. However, a greater understanding of how the different microbes interact with one another is needed, since this will lead to a better understanding of the intricate relationship between the microbes. Subsequently, the impact that the various environmental factors have on the microbial relationship of Kepi grains will also be more clear. However, the data obtained in this study clearly showed that the method by which Kepi is produced, the origin of Kepi grains and the method of Kepi grain preservation changes the relationship between the microbes constituting the grains to such an extent that a different microbial community is assembled.

Very few studies have been done in which propionibacteria have been successfully incorporated into Kepi. An understanding of the intricate relationship between the microbes present in Kepi grains will facilitate the successful incorporation of microbes such as propionibacteria into Kepi grains. Detection of these added microbes using a molecular method, as used in this study, will enable the success of the enrichment processes to be measured very rapidly and effectively.

The results obtained in this thesis have shown that there are many factors to consider when determining the microbial community of a fermented product such as Kepi grains. It is suggested that traditional methods be used together with newer methods to prevent any misleading results. However, there is still much to be learned about the microbial community of Kepi grains and many questions must still be answered.

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